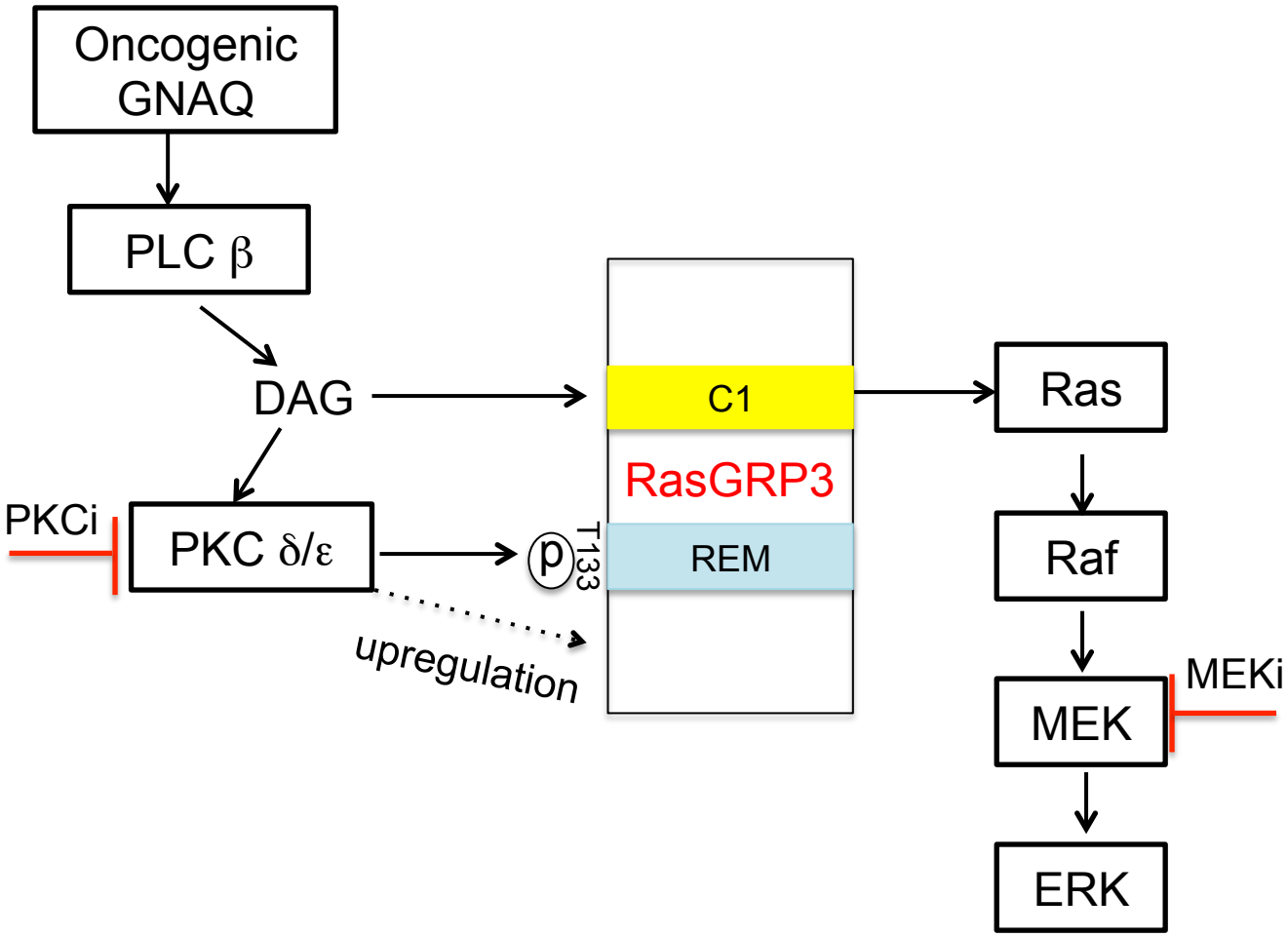


Highlights

- Out of over 10 different PKC isoforms, PKC δ and PKC ε are identified as effectors that mediate activation of the MAP-kinase pathway downstream of mutant GNAQ in uveal melanoma.
- The RAS exchange factor RasGRP3 is markedly upregulated in uveal melanoma cells and is an essential effector that links oncogenic Gq signaling to MAPK signaling by three mechanisms: DAG binding to its C1 domain, phosphorylation at T133 by PKC δ and ε, and increased expression levels also mediated by PKC δ and ε.
- PKC δ, PKC ε and RasGRP3 are novel therapeutic targets for uveal melanoma.

RasGRP3 mediates MAPK pathway activation in GNAQ mutant uveal melanoma

Xu Chen[1*], Qiuxia Wu[1], Philippe Depeille[2], Peirong Chen[1], Sophie Thornton[3], Helen Kalirai[3], Sarah E. Coupland[3], Jeroen P Roose[2], Boris C Bastian[1*]

[1]Departments of Dermatology and pathology, and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA 94143

[2]Department of Anatomy, University of California, San Francisco, San Francisco, CA 94143

[3] Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool, United Kingdom

* Correspondence: Xu.Chen@ucsf.edu (X.C.) or Boris.Bastian@ucsf.edu (B.B.)

Highlights

- Out of over 10 different PKC isoforms, PKC δ and PKC ε are identified as effectors that mediate activation of the MAP-kinase pathway downstream of mutant GNAQ in uveal melanoma.
- The RAS exchange factor RasGRP3 is markedly upregulated in uveal melanoma cells and is an essential effector that links oncogenic Gq signaling to MAPK signaling by three mechanisms: DAG binding to its C1 domain, phosphorylation at T133 by PKC δ and ε, and increased expression levels also mediated by PKC δ and ε.
- PKC δ, PKC ε and RasGRP3 are novel therapeutic targets for uveal melanoma.

Summary

Constitutive activation of Gαq signaling by mutations in GNAQ or GNA11 occurs in over 80% of uveal melanomas (UM) and activates the MAP-kinase signaling cascade. Protein kinase C (PKC) has been implicated as a link, but the mechanistic details remained unclear. We identified PKC δ and PKC ε as required and sufficient to activate MAPK in GNAQ mutant melanomas. We find that MAPK activation depends on the presence of Ras and is caused by RasGRP3, a Ras exchange factor that is significantly and selectively overexpressed in response to GNAQ/11 mutation in UM. RasGRP3 activation occurs via PKC δ- and ε-dependent phosphorylation as well as PKC-independent, DAG-mediated membrane recruitment, possibly explaining the limited effect of PKC inhibitors to durably suppress MAP-kinase signaling in UM. The findings nominate RasGRP3 as a possible therapeutic target for cancers driven by oncogenic GNAQ/11.

Significance

Uveal melanoma (UM) is the most lethal form of melanoma with a 10-year survival rate of approximately 50%. Once metastatic, no effective treatment options exist as current targeted therapy approaches and immune checkpoint blockade regimens are less effective than in cutaneous melanomas. The identification of PKC δ and PKC ε as critical effectors downstream of an activated Gαq signaling pathway highlights them as specific targets for therapy. The critical role of RasGRP3 as an oncogenic effector and transcriptional target downstream of GNAQ/11 may offer additional opportunities for the development of treatments.

Introduction

Uveal melanoma (UM) is the most common intraocular malignancy in adults and cannot be effectively treated once metastatic (Singh et al., 2005). UM is a genetically and biologically distinct type of melanoma with a significantly lower 10-years survival than cutaneous melanoma. It originates from melanocytes of the choroidal plexus, ciliary body or iris of the eye and does not harbor mutations in BRAF, NRAS and KIT that

prevail in cutaneous melanomas arising from intraepithelial melanocytes of the skin or mucosa. Instead, UM harbor mutually exclusive mutations in GNAQ, GNA11, PLCB4 or CYSLTR2 (Johansson et al., 2016; Moore et al., 2016; Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). GNAQ and GNA11 encode closely related, large GTPases of the $G\alpha_q$ family, which are α subunits of heterotrimeric G proteins that operate downstream of G protein–coupled receptors (GPCRs). Relevant $G\alpha_q$ coupled receptors in melanocytes include endothelin and WNT receptors, which play a critical role in melanocyte differentiation and survival and have been associated with invasion and metastasis in melanoma, and the cysteinyl leukotriene receptor 2 (CYSLTR2) (Dissanayake et al., 2007; Moore et al., 2016; Sheldahl et al., 1999; Shin et al., 1999). Approximately 95% of GNAQ and GNA11 mutations in melanoma affect codons 209 (Q209) of the G-proteins with only 5% affecting codon 183 (R183) in the Ras-like domain. The respective mutations result in complete or partial loss of GTPase activity, thereby locking GNAQ/11 into its active protein, GTP-bound confirmation, resulting in a dominant acting oncogene that transforms melanocytes (Van Raamsdonk et al., 2009; Van Raamsdonk et al., 2010). Similar to the mutations in RAS oncoproteins, the defects in GNAQ or GNA11 GTPases are difficult to target directly, making it important to delineate the oncogenic effector pathways downstream to identify opportunities for targeted therapy. Recently, mutations in the CYSLTR2, a Gq-coupled GPCR, and the downstream effector of Gαq PLCB4, encoding a phospholipase Cβ (PLC β) isoform, have been reported in the small percentage of UMs without GNAQ or GNA11 mutations(Johansson et al., 2016; Moore et al., 2016).

MAP-kinase pathway activation has been shown as one contributing factor to GNAQ mediated oncogenesis (Van Raamsdonk et al., 2009). However, how exactly mutant GNAQ/11 relays signals to the MAPK pathway in UM remains to be clarified. Recent studies have demonstrated MAP-kinase pathway activation is at least in part mediated by protein kinase C (PKC)(Chen et al., 2014; Wu et al., 2012). PLC β, a direct downstream effector of mutant GNAQ/11, hydrolyzes the membrane phospholipid phosphatidylinositol 4,5-bisphosphate to release two potent second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). DAG provides a docking site

within the inner leaflet of the plasma membrane for enzymes, including specific PKC isoforms (Hubbard and Hepler, 2006) and IP3 releases calcium from the smooth endoplasmic reticulum (ER). Calcium also activates some PKC isoforms. The PKC family consists of at least 10 serine/threonine kinases, which are subdivided into classic, novel and atypical isoforms (Griner and Kazanietz, 2007). The classical PKC isoforms (α, βI, βII, γ) can be activated by both DAG and calcium, while the novel PKCs (δ, ε, θ, η) are only DAG-dependent. By contrast, the atypical PKCs (ζ, ι) are not responsive to DAG or calcium. Which specific PKC isoforms mediate the activating effect on the MAPK pathway in the context of GNAQ or GNA11 mutations remains unclear. Recent studies have also demonstrated that mutant GNAQ/11 promote uveal melanoma tumorigenesis by activating YAP independent of PLC β(Feng et al., 2014; Yu et al., 2014). Furthermore, prior studies have shown that PKC inhibition alone in UM cell lines is not sufficient to completely suppress MAPK signaling (Chen et al., 2014), suggesting that PKC-independent effectors may be involved that mediate MAPK signaling in GNAQ/11 mutant cells. Alternative effectors that may connect PLCβ-generated IP3 and DAG to MAPK signaling in UM and have recently been implicated in various types of cancer are members of the RasGRP family of RasGEFs (Ras guanine nucleotide exchange factors) (Ksionda et al., 2013). Studies in $BRAF^{V600E}$ melanomas suggest that therapeutically meaningful responses in patients can only be expected if marked suppression of the MAPK pathway is achieved (Bollag et al., 2010; Flaherty et al., 2012). To achieve this goal in UM, a more refined understanding of the connection between MAPK signaling and GNAQ/11 mutant in uveal melanoma is required to develop more effective targeting strategies.

Here, we identified PKC δ and PKC ε as key PKC isoforms to activate MAPK signaling in GNAQ mutant melanoma and identified RasGRP3 as a critical signaling nodule linking oncogenic GNAQ to Ras/Raf/MEK/ERK pathway via multiple mechanisms, implicating PKC δ and PKC ε and RasGRP3 as possible therapeutic targets for cancers driven by oncogenic GNAQ/11.

Results

PKC δ and PKC ε are the critical PKC isoforms that mediate MAP-kinase signaling in uveal melanoma cells with GNAQ/11 mutation

Oncogenic GNAQ activates MAPK in part via PKC (Chen et al., 2014). However, which of the numerous isoforms are involved in this signaling is not entirely resolved. The significant homology among the different isoforms α, βI, βII, γ, δ, ε, θ, η, ζ, ι poses challenges for their specific identification. In order to overcome this limitation we tested the specificity of commercially available PKC antibodies by transfecting HA-tagged full length PKC isoforms or their catalytic subunits (Figure S1A and S1B) into 293FT cells and identified a panel of specific antibodies that was used to screen a panel of melanoma cells with or without GNAQ/11 mutations. As shown in Figure 1A, five PKC isoforms (PKC α, PKC δ, PKC ε, PKC ζ and PKC ι) were consistently expressed throughout all 6 melanoma cells tested (MEL202, 92-1, OMM1.3, MEL270 with GNAQ mutations and OMM-GN11, UPMD-1 with GNA11 mutations). These isoforms were also expressed at similar levels in most of the melanoma cell lines tested that had mutations other than GNAQ or GNA11 and partly originated from cutaneous melanomas (C8161, MM415, MM485, SK-MEL-28). This expression pattern is concordant with prior reports for melanoma cell lines (Denning, 2012; Oka and Kikkawa, 2005). By contrast, PKC βI, PKC θ, PKC η and PKC γ expression was not detected in any of the 13 melanoma cells, mostly consistent with previous studies(Denning, 2012; Gilhooly et al., 2001; Oka and Kikkawa, 2005; Selzer et al., 2002), although PKC η expression has been described in some melanoma cells and tissues (Oka and Kikkawa, 2005). While PKC β II showed differential expression between melanoma cell lines with and without GNAQ/11 mutations, it was not expressed in all GNAQ/11 mutant cell lines, ruling it out as a universal effector downstream of mutant GNAQ/11. While PKC ζ and ι, where found to be expressed in GNAQ/11 mutant cell lines, they are classified as atypical PKCs as they are not activated by either calcium or DAG, making them unlikely candidate effectors of mutant Gαq. By contrast, PKC α, δ and ε, which were consistently expressed in all uveal melanoma cell lines with GNAQ or GNA11 mutations, are candidates to mediate MAPK signaling in GNAQ mutant melanomas.

In order to determine which of these PKC isoforms mediate MAPK signaling in GNAQ/11 mutant cells, we performed siRNA mediated knock down of PKC isoforms (PKC α, δ, ε as well as ζ used as control) alone or in combination in 3 GNAQ mutant cell lines (92-1, OMM1.3, and MEL202) and examined MAPK signaling at the level of pMEK and pERK. As shown in Figure1B and Figure S1C, knockdown of either PKC δ or ε alone resulted in partial inhibition of pMEK and pERK, whereas knockdown of PKC α and PKC ζ had no significant effect. Combined knockdown of PKC δ and ε inhibited pMEK and pERK levels similar to knockdown of GNAQ itself. Similar results were observed in the GNA11 mutant cell line UPMD-2 (Figure S1C). In contrast to the suppressive effect on MAP-kinase pathway activation in GNAQ mutant cell lines, knockdown of GNAQ, PKC δ and PKC ε singly or in combination had no effect in three melanoma cell lines without GNAQ mutations (C8161 from cutaneous origin, MUM2C and MEL285 from uveal origin) (Figures 1C and S1D). By contrast, knockdown of PKC βII in 92-1 with GNAQ mutation (Figure 1B) and OMM-GN11 and UPMD-2 cell lines with GNA11 mutation (Figure S1E) had no effect on pMEK and pERK.

To establish an initial connection between specific PKC isoforms and mutant GNAQ, we co-transfected PKC isoforms (α, δ, ε, ζ, ι) together with $GNAQ^{Q209L}$ into the 293FT model cell line. As shown in Figure1D, only PKC δ and PKC ε strongly synergized with $GNAQ^{Q209L}$ in activating the MAP-kinase pathway. We confirmed that the synergistic effect was dependent on the kinase activity of PKC δ and ε, by using kinase-dead mutants of PKC δ and ε (Figure S2A). Furthermore, only $GNAQ^{Q209L}$ but not wild type GNAQ synergized with PKC δ and ε, indicating that the synergy depended on active GNAQ (Figure S2B).

PKC is recruited by DAG to the plasma membrane which contributes to its activation. In normal melanocytes, both PKC α and PKC δ were localized in the cytosolic fraction, while PKC ε were localized in both cytosolic and membrane fractions (Figure S2E). After stimulation with the stable DAG analogue TPA, the PKC isoforms α, δ, and ε but not ζ translocated to the membrane (Figure S2G). In uveal melanoma cell lines with GNAQ/11 mutations, PKC α and PKC ζ were localized in the cytosolic fraction, while a considerable portion of the PKC δ and PKC ε signal was localized at the

membrane (Figure S2F). These data further support the notion that the main active isoforms in the context of GNAQ/11 mutations are PKC δ and PKC ε.

As previously shown, cell proliferation of GNAQ mutant UM cells depends on GNAQ and PKC as knockdown of GNAQ or PKC inhibition suppress cell proliferation (Chen et al., 2014; Khalili et al., 2012; Wu et al., 2012). To determine whether this inhibition is mediated by PKC δ and PKC ε, we investigated their contribution by knocking them down both singly and in combination. Combined knockdown of PKC δ and PKC ε but not PKC α reduced cell proliferation similar to knockdown of GNAQ in GNAQ mutant cell lines (92-1 and OMM1.3), while the knockdown had no effect in cell lines without GNAQ mutations (Figure 1E). Similar results were obtained in the GNA11 mutant cell line (UPMD-2) (Figure 1E). We confirmed that expression levels of the targeted genes remained suppressed during the course of these experiments (Figure S2C and S2D). Taken together, these experiments indicate that PKC δ and PKC ε are essential for MAPK signaling and proliferation of UM cell lines with GNAQ mutations but not in related cell lines without GNAQ mutations.

Ras is required for oncogenic GNAQ to activate the MAP-kinase pathway

To determine at which level mutant GNAQ activates the MAP-kinase pathway, we investigated whether the activation depended on Ras. We found that 293FT cells transfected with $GNAQ^{Q209L}$ had increased levels of Ras-GTP compared to controls, which could be further increased by co-transfection of PKC δ and ε compared to co-transfection of GFP (Figure 2A), PKC α, or ζ (Figure S2H). TPA also increased Ras-GTP levels. All three Ras isoforms (Hras, Kras and Nras) were activated by oncogenic GNAQ (Figure 2B). Only simultaneous knockdown of all three Ras isoforms, but not knockdown of individual isoforms, decreased pMEK and pERK levels comparable to knockdown of GNAQ and inhibited cell proliferation (Figures 2C and S2I) indicating that activation of MAPK signaling in GNAQ mutant cells requires the presence of Ras, with no specific preference of any specific Ras family member.

The Ras-GEF RasGRP3 is implicated as a signaling module in uveal melanoma

To determine how mutant GNAQ activates Ras/MAPK signaling, we compared gene expression profiles between five GNAQ or GNA11 mutant melanoma cells ($GNAQ^{mt}$: 92-1, MEL202, OMM1.3; $GNA11^{mt}$: UPMD-1, OMM-GN11) and five GNAQ/11 wildtype melanoma cells with either NRAS (SK-MEL-2, MM415, MM485) or BRAF mutations (SK-MEL-5 and MUM2C). As shown in Figure 3A, RasGRP3, a Ras-guanyl nucleotide exchange factor (RasGEF), ranked at the top of 487 differentially expressed genes between (cut off: p value <0.05; fold change >2 or <-2). In addition, RasGRP3 spiked our particular interest because Ras-MAPK activation via RasGRP3 is regulated by the 2nd messenger DAG and by phosphorylation by PKC(Ksionda et al., 2013). Quantitative reverse transcriptase (RT)-PCR experiments confirmed that RasGRP3 mRNA is markedly up-regulated (>100 fold) in melanoma cell lines with GNAQ or GNA11 mutations, compared to normal melanocytes or melanoma cell lines with other mutations. Other RasGRP family members as well as the RasGEF SOS1 did not show any increased expression levels in melanoma cell lines with GNAQ or GNA11 mutations or showed differential expression differences in these cells (Figure S3A). A comparison of RasGRP3 mRNA levels across TCGA datasets (Figure 3C) revealed that RasGRP3 expression in uveal melanoma tissues is consistently increased and considerably higher than in other cancer types, including cancers reported to have elevated RasGRP3 expression, such as breast cancer (Nagy et al., 2014), prostate cancer (Yang et al., 2010), lymphoma(Teixeira et al., 2003) and cutaneous melanoma(Yang et al., 2011). Interestingly, the four samples in the 478 samples of cutaneous melanoma samples that harbor GNAQ or GNA11 hotspot mutations, also had elevated RasGRP3 mRNA with levels comparable to those present in uveal melanoma (Figure 3D). These data suggest that higher expression of RasGRP3 is specifically associated with the presence of oncogenic GNAQ/11.

Western blotting with validated antibodies (Figure S3B) demonstrated that the increased RasGRP3 mRNA levels lead to elevated protein levels (Figure 3E and Figure S3C). Similarly, the lymphoblastoid cell line Ramos B (Teixeira et al., 2003) and the cutaneous melanoma cell lines SK-MEL-5 and UACC257 (Yang et al., 2011) have been reported to express elevated RasGRP3 levels. We found that both RasGRP3 and p-RasGRP3(T133) protein levels in melanoma cell lines with GNAQ mutations were

significantly higher than in cell lines without GNAQ mutations, including SK-MEL-5 and UACC257 (Figure S3D). We also examined RasGRP3 protein expression in formalin-fixed, paraffin-embedded (FFPE) uveal melanoma tissues by immunohistochemistry (IHC). As shown in Figure S3E, two conjunctival melanoma cell lines that had no GNAQ/11 mutations (CRMM1 and CRMM2) had no detectable expression of RasGRP3 protein by western blot and immunohistochemistry (Figure 3F), while uveal melanoma cell lines with GNAQ mutation (92-1, MEL270) or GNA11 mutation (OMM1) showed strong RasGRP3 immunoreactivity (Figure 3F and Figure S3F). RasGRP3 protein expression was also detected by immunohistochemistry in uveal melanoma tissues with GNAQ and GNA11 mutations (Figure 3G).

Elevated RasGRP3 expression and RasGRP3 phosphorylation is a consequence of mutant GNAQ signaling and is mediated by PKC δ and PKC ε

We next investigated the connections between GNAQ and RasGRP3 and noted that knockdown of GNAQ diminished not only p-RasGRP3 levels but also decreased total RasGRP3 (Figure 4A). Conversely, expression of $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ but not $BRAF^{V600E}$ in human melanocytes significantly increased total RasGRP3 and pRasGRP3 levels (Figure 4B). The upregulation of RasGRP3 expression depended on PKC activity. Treatment of OMM1.3 cells with PKC inhibitor AEB071 (500nM) suppressed RasGRP3 levels in a time-dependent manner, starting after 10h, peaking at 24h after drug administration (Figure 4C). This time course contrasts with the suppression of RasGRP3 phosphorylation by AEB071, which was detectable already at 2h and peaked at 10h. Maximal pERK inhibition was observed at about 24 h, probably reflecting the combined effect of inhibition of RasGRP3 phosphorylation and downregulation of its expression. Similar results were observed in the 92-1 cell line (Figure S4A). The effect of PKC inhibition on RasGRP3 expression levels and p-RasGRP3 levels was dose dependent in three different GNAQ mutant cell lines incubated with two different PKC inhibitors, AEB071 and AHT956 for 24h (Figure 4D and Figure S4B), but had no effects on RasGRP3 expression while inhibiting pRasGRP3 in melanoma cell lines with $Braf^{V600E}$ or NRAS mutations (Figure S4C), which have very weak expression of RasGRP3 compared to GNAQ mutant cells. TPA

increased RasGRP3 expression levels and RasGRP3 phosphorylation in a time dependent manner in human melanocytes, with no significant effects on RasGRP1 and SOS1 (Figure 4E and Figure S4E). TPA also increased RasGRP3 expression in human melanoma cells with BRAF mutations (Figure S4D). These data indicate that activation of PKC in melanocytic cells results in upregulation of RasGRP3 expression as well as T133 phosphorylation of RasGRP3, and that the markedly elevated protein and phosphorylation levels found in GNAQ mutant melanoma cells are a consequence of Gαq-mediated upregulation of PKC activity. Among the four PKC isoforms PKCα, PKCδ, PKCε, PKCζ, which we found to be invariably expressed in GNAQ or GNA11 mutant UM cell lines, only PKCδ and PKCε robustly phosphorylated T133 of RasGRP3, whereas PKC α and PKC ζ had weaker effects with no significant consequences on MAPK signaling (Figure 4F and Figure S4F). Combined knockdown of PKC δ and PKC ε suppressed RasGRP3 expression and RasGRP3 phosphorylation and resulted in maximal inhibition of pERK and pMEK compared to non-target siRNA control, while knockdown of individual PKC isoforms and combined knockdown of PKCα with δ or ε had no significant effect (Figure 4G).

Next we investigated the mechanism of RasGRP3 upregulation. We ruled out a positive feedback loop involving MAPK activation and RasGRP3 regulation by suppressing MAP-kinase signaling using the two different MEK inhibitors PD0325901 and Trametinib (Figure 4H and Figure S4G). The YAP1 pathway has been shown to play an important role in GNAQ mutant melanoma development(Feng et al., 2014; Yu et al., 2014). Knock-down of YAP1 did not affect RasGRP3/MAPK regulation and activation (Figure S5A). Combined inhibition of the YAP1 and PKC/MAPK pathways also neither synergistically suppressed MAPK signaling nor increase cleaved PARP levels, compared to combined inhibition of PKC and MEK (Figure S5B-C). The YAP1 inhibitor verteporfin or the MET inhibitor INC280 did not increase the effects of the PKC inhibitor AEB or the MEK inhibitor 162 (Figure S5F). The oncogene tyrosine kinase receptor MET was found to be highly expressed in UM cell lines with GNAQ/11 mutation in our microarray experiment (Figure 3A). However, knock-down of MET or inhibition with the MET inhibitor INC280 had no effect on RasGRP3 and pRasGRP3 levels nor

pERK in UM cell lines (Figure S5D-E). These data indicate that neither the YAP1 pathway nor MET signaling is involved in the upregulation of RasGRP3.

Some cancers have RasGRP3 amplifications (Figure S6D), but we found no incident of amplification of RasGRP3 or PKC δ and PKC ε in UM cell lines (Figure S6A and S6B) and UM tissue samples (Figure S6C). To investigate whether the upregulation occurs at the transcription level, we knocked-down GNAQ and found that it decreased RasGRP3 mRNA levels in the GNAQ mutated UM cell lines OMM1.3 and 92-1 (Figure S6E). By contrast, the knockdown had no effect on the RASGEF SOS1. The PKC inhibitors AEB071 and AHT956 also both decreased RasGRP3 mRNA, also with no effect on SOS1 mRNAs (Figure S6G). Conversely, overexpression of $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ in melanocytes increased RasGRP3 mRNA (Figure S6F). Similarly, TPA increased RasGRP3 mRNA in both melanocytes and in the BRAF-mutant melanoma cell line UACC257 (Figure S6H). All together, these data suggest RasGRP3 regulation in the context of GNAQ mutations occurs transcriptionally and involves PKC signaling.

RasGRP3 mediates MAPK activation in GNAQ mutant melanoma cells

The consistently elevated expression levels implicated RasGRP3 as a candidate protein involved in activation of Ras signaling downstream of mutant GNAQ/11. Knockdown of RasGRP3 in GNAQ mutant melanoma cell lines significantly reduced levels of pMEK and pERK but not pAKT (Figure 5A and Figure S7A) but had no effect in other melanoma cell lines (Figure 5B). Similar results were obtained with individual siRNAs instead of siRNA pools (Figure S7B). Knockdown of other RasGRP isoforms had no effect on pMEK and pERK (Figure S7C), but the protein levels of RasGRP1, RasGRP2 and RasGRP4 were already too low to be detectable even before knockdown. Knockdown of SOS1 only slightly reduced pMEK and pERK levels (Figure S7D). Knockdown of either GNAQ or RasGRP3 also decreased RasGTP levels in GNAQ mutant uveal melanoma cell lines (Figure S7E).

siRNA mediated knockdown of RasGRP3 also significantly decreased proliferation of GNAQ mutant cells during a five day proliferation assay, whereas it had no significant effect on other melanoma cell lines (Figure 5C). We verified that RasGRP3 expression remained suppressed during the course of the experiment (Figure

S7F). Stable shRNA-mediated knockdown of RasGRP3 also markedly reduced tumor growth in a mouse xenograft model of the GNAQ mutant cell line 92-1 (Figure 5D-F).

To further confirm that RasGRP3 modulates MAPK signaling in the setting of GNAQ mutation, we co-transfected 293FT cells with $GNAQ^{Q209L}$ combined with individual RasGRP isoforms and examined their effects on MAPK signaling. As shown in Figure 5G, RasGRP1, RasGRP3 and RasGRP4 alone but not RasGRP2 induced pERK , while only co-transfection $GNAQ^{Q209L}$ with RasGRP1 or RasGRP3 potentiated MAPK signaling as evidenced by markedly increased pMEK and pERK level. In contrast, co-transfection $GNAQ^{Q209L}$ with RasGRP2 had no synergistic effect on MAPK signaling, consistent with the notion that RasGRP2 activates Rap but not Ras (Ksionda et al., 2013). We ruled out that these differences were due to variation in the expression levels of the transfection constructs using antibodies against the myc tag of RasGRP isoforms. $GNAQ^{Q209L}$ increased pRasGRP1 (T184) and pRasGRP3(T133) levels in RasGRP1- and RasGRP3- expressing cells, respectively, indicating that oncogenic GNAQ can in principle activate both RasGRP1 and RasGRP3. Our data indicate that in the context of uveal melanoma cells RasGRP3 is the relevant effector and is upregulated at the mRNA level, whereas RasGRP1 is not expressed (Figure 3E).

RasGRP3 is activated by PKC phosphorylation and DAG-mediated membrane recruitment

Next we performed a series of experiments to examine the mechanisms by which RasGRP3 activates MAPK signaling in uveal melanoma cells. In B cells, it has been shown that PKC can phosphorylate RasGRP3 at T133, located in its Ras exchange motif (REM) (Zheng et al., 2005). A phosphorylation site mutant $RasGRP3^{T133A}$ substantially reduces but does not abrogate all RasGRP3 activity in B cells (Aiba et al., 2004). To determine the role of T133 phosphorylation in the context of mutant GNAQ/11, we introduced $RasGRP3^{T133A}$ into 293FT cells. RasGRP3 ^{T133A} also was less effective in synergizing with $GNAQ^{Q209L}$ in activating the MAP-kinase pathway (Figure 6A) and Ras (Figures S7G) than wild type RasGRP3. We confirmed that the PKC isoform expression patterns in 293FT cells and UM cells are comparable, with the exception of PKC θ, which is only expressed in 293FT cells (Figure S7H). These data

indicate that PKC phosphorylation at T133 also contributes to RasGRP3 mediated Ras/MAPK signaling in the context of mutant GNAQ. However, the phosphorylation site mutant of RasGRP3 had a residual activating effect on the MAP-kinase pathway, indicating some RasGRP3 activity may be PKC independent. To resolve this, we inhibited PKC activity using the pan-PKC inhibitor AEB071 in 293FT cells transfected with combinations of mutant or wild type RasGRP3 with mutant GNAQ (Figure 6B). At a concentration of 1μM AEB071 was able to completely abrogate T133 phosphorylation of RasGRP3, consistent with prior studies showing complete extinction of PKC activity at this concentration(Chen et al., 2014). By contrast, AEB071 had no significant effect on MAP-kinase pathway activation of wild type RasGRP3 or RasGRP3^{T133A} in the absence of mutant GNAQ, indicating that this residual activity was PKC independent. As a control, addition of a MEK inhibitor, PD0325901 in this experiment blocked all MAPK activation (Figure 6C). These data indicate that PKC phosphorylation at T133 contributes to RasGRP3 mediated MAPK signaling, but pinpoints a residual activating effect of RasGRP3 that does not rely on phosphorylation at T133 or PKC activity, which is consistent with the reported work in B cells (Aiba et al., 2004).

RasGRP3 is recruited to the inner leaflet of the plasma membrane by binding DAG via its C1 domain(Lorenzo et al., 2001). In order to determine the role of membrane recruitment of RasGRP3 on MAPK signaling, we generated C1 domain deletion constructs of RasGRP3. As shown in Figure 6D, deletion of the C1 domain of RasGRP3 significantly reduces the ability of mutant GNAQ to activate the MAP-kinase pathway and the RasGRP3 activity could be further reduced by mutating the T133 phosphorylation site. The effect of C1 deletion could be completely rescued by adding Fyn or Src membrane binding motifs(Chen and Resh, 2001) to the RasGRP3 C1 deletion mutants (Figure 6E), indicating that C1-mediated membrane binding is essential for GNAQ-mediated activation of MAP-kinase signaling. All together, these data demonstrate that membrane binding via the C1 domain and T133 phosphorylation cooperate in RasGRP3-mediated activation of MAPK signaling in the setting of GNAQ mutations.

Discussion

Constitutive activation of the MAPK pathway (RAS/RAF/MEK/ERK) is common in cutaneous melanoma and typically occurs through mutations and/or amplification of signaling modules such as BRAF or NRAS. The MAP-kinase pathway is also activated in UM, but mutations of these specific genes are conspicuously absent. Instead, this disease appears to be defined by mutations of the Gαq pathway. The mechanism how these mutations result in activation of MAP-kinase signaling is incompletely understood. Prior studies have shown Gαq mediated activation of MAPK through the kinases Pyk2 and src-mediated activation of Ras in PC12 cells and HEK293 cells (Della Rocca et al., 1997; Dikic et al., 1996; Lev et al., 1995), an effect that is PKC independent. Gαq has also been shown to activate the MAP-kinase pathway independent of RAS through RasGRP2 and Rap1 mediated activation of B-Raf in PC12 cells(Guo et al., 2001). Another RAS-independent mechanism of MAP-kinase pathway activation by Gαq involving PKC-mediated phosphorylation of Raf-1 has been shown using NIH3T3 cells (Kolch et al., 1993), COS-1 cells (Ueda et al., 1996) and COS-7 cells (Schonwasser et al., 1998) as models.

We show that in the context of oncogenic GNAQ signaling found in UM, the activation of the MAP-kinase pathway occurs through a different mechanism. We find that Ras is required for GNAQ mediated MAPK activation, and identify a signaling module that involves PKCδ and ε and RasGRP3, which is required and sufficient to activate Ras/MAPK signaling.

Among the more than 10 different PKC isoforms we found that irrespective of GNAQ or GNA11 mutation status, PKC α, δ, ε, ζ were ubiquitously expressed in melanoma cells, while PKC ι was only weakly expressed, mostly consistent with published reports. Among the consistently expressed isoforms we identified PKC δ and PKC ε as the main functionally important isoforms that mediate MAPK signaling in uveal melanoma cells. Only knock-down of PKC δ or PKC ε but not other PKC isoforms significantly reduced MAPK signaling in GNAQ mutant melanoma cells. Only Combined

depletion of PKC δ and PKC ε but not combined knockdown of either PKC ζ with δ or ε (Figure 1B-C and Figure S1C-D), or PKC α with δ or ε (Figure 4G) extinguished MAP-kinase pathway signaling and inhibited proliferation GNAQ mutant melanoma cell lines but had no such effect on melanoma cell lines with other mutations (Figure 1E). Concordantly, co-transfection $GNAQ^{Q209L}$ with PKC δ or PKC ε but not PKC α, PKC ζ, and PKC ι potentiated MAPK signaling in 293FT cells (Figure 1D).

We identified PKC δ and PKC ε as the main effector isoforms downstream of oncogenic GNAQ/11 that activate MAPK signaling. The lack of MAPK activation by PKC α is of interest as it, like PKC δ and PKC ε, also contains a DAG binding domain. However, we found PKC α localized mainly in the cytoplasm in uveal melanoma cell lines, while a considerable fraction of PKC δ and PKC ε were localized at the membrane, consistent with their activation in this setting.

RasGRP3 belongs to family with four related RAS GTP exchange factors (GEFs) RasGRP1-4, which all contain a REM (Ras exchange motif)-Cdc25 domain that mediates Ras activation and a C1 domain that mediates membrane localization by binding DAG (Ebinu et al., 1998; Ksionda et al., 2013; Stone, 2011). RasGRP proteins display tissue-specific expression patterns: RasGRP1 is primarily expressed in the cerebral cortex, cerebellum, T cells and to a lesser extent in B cells; RasGRP2 is expressed in striatum and platelets and their precursors; RasGRP3 is highly expressed in B cells, macrophages and endothelial cells; whereas RasGRP4 is restricted to mast cells, thymocytes and neutrophils. Recent studies have shown that RasGRP3 plays an important functional role in the formation and progression of a variety of human cancers (Nagy et al., 2014; Yang et al., 2010; Yang et al., 2011).

We show that the significantly increased expression level of RasGRP3 in UM is a direct consequence of Gαq signaling and that Gαq signaling not only increases RasGRP3 transcription in a PKC-dependent manner but activates RasGRP3 by two independent mechanisms: membrane recruitment and phosphorylation by PKC δ and PKC ε.

RasGRP3 is reported to be expressed at elevated levels in some human cancers such as Burkitt's lymphoma, metastatic prostate cancer, human breast cancer and BRAF mutant melanoma(Nagy et al., 2014; Teixeira et al., 2003; Yang et al., 2010;

Yang et al., 2011). Here we show that the expression level of RasGRP3 in GNAQ or GNA11 mutant melanoma cell lines and human uveal melanoma tissues, by far exceeds the expression levels of the previously reported tissue types indicating a specific role of this protein in uveal melanoma. Our results indicate that GNAQ signaling and PKC δ and ε are involved in upregulating RasGRP3 expression, and that this effect is restricted to melanocytes as mutant GNAQ did not increase RasGRP3 expression levels in 293FT(data not shown). Other RasGRP family members were not expressed at significant levels in UM cell lines. The mechanistic details behind this apparently Rasgrp3- and cell type- specific regulation remain to be elucidated.

Phosphorylation of RasGRP3 at Threonine 133 by PKC enhances RasGRP3's ability to maximally activate Ras signaling in antigen receptor-stimulated B cells, and in that context is suggested to be mediated by PKC θ (Aiba et al., 2004)and PKC β(Zheng et al., 2005). We confirm the critical role of RasGRP3 phosphorylation at T133 in the setting of GNAQ mutations with PKC δ and PKC ε as the relevant PKC isoforms for this modification in UM. We show that mutating the PKC phosphorylation site of RasGRP3 (T133A) partially but not completely attenuated RasGRP3 mediated MAPK signaling. The residual RasGRP3 activity depended on its DAG binding domain. These findings indicate the existence of two independent mechanisms that activate RasGRP3 in UM, one depends on PKC phosphorylation at T133 and the other requires DAG. DAG here most likely serves to recruit RasGRP3 to the plasma membrane, as the DAG-binding domain could be substituted by either Fyn or Src membrane target motifs. This PKC-independent activity may explain why PKC inhibition fails to induce sustained suppression of MAPK signaling in GNAQ mutant melanomas(Chen et al., 2014). In line with our findings, both the catalytic domain and the C1 domain of RasGRP3 are required in BCR-mediated Ras activation(Oh-hora et al., 2003).

Our finding that GNAQ mediates MAPK signaling via PKC δ and PKC ε in UM may have important clinical implications and provide an opportunity for refined therapeutic targeting. To date, clinical studies on UM utilize pan-PKC inhibitors including AEB071, but more specific compounds targeted to the PKC δ and PKC ε hold the promise of an improved therapeutic index. The key role of RasGRP3 in mediating signals to the MAP-kinase pathway implicates it as a novel therapeutic target.

Experimental Procedures

Plasmids and reagents

The GNAQ and GNA11 constructs have been previously described (Chen et al., 2014). HA-tagged PKC isoforms full-length cDNA constructs (α, βII, δ, ε, γ, η, ζ, ι), HA-tagged catalytic domains cDNAs (α, βII, δ, ε) and HA-tagged kinase dead cDNAs (δ, ε) are gifts from Bernard Weinstein(Soh and Weinstein, 2003) and purchased from Addgene (Cambridge, MA). Myc tagged human RasGRP1, RasGRP2, RasGRP3 and RasGRP4 cDNAs were cloned into pEF6 vector. RasGRP3-T133A construct was generated by site-directed mutagenesis using Quikchange II kit (Agilent Technologies, Santa Clara, CA) following the manufacturer's instruction. C1 domain is deleted to create two C1 domain deletion mutant constructs (RasGRP3-ΔC1 and RasGRP3-T133A-ΔC1) by using overlap extension PCR. PCR products were inserted into pEF6 vector following EcoRI and XbaI digestion. RasGRP3- ΔC1 and RasGRP3-T133A- ΔC1 were fused at their N-terminus to 20 N-terminal Src or 16 N-terminal Fyn amino acids(Chen and Resh, 2001) by using PCR method, resulting in generation of Src-RasGRP3- ΔC1, Src-RasGRP3- T133A-ΔC1, Fyn-RasGRP3- ΔC1, and Fyn-RasGRP3-T133A-ΔC1. The primers used for Src fusion constructs are as follows: 5'-GGTGGAATTCATGGGGAGTAGCAAGAGCAAGCCTAAGGACCCCAGCCAGCGCCGGCGCAGCCTGGAGCCAGGATCAAGTGGCCTTGGG-3' and 5'-ATCCTCTAGAGCCATCCTCACCATC -3'. The primers used for Fyn fusion constructs are: 5'-GGTGGAATTCATGGGGAGTAGCAAGAGCAAGCCTAAGGACCCCAGCCAGCGCCGGCGCAGCCTGGAGCCAGGATCAAGTGGCCTTGGG-3' and 5'-ATCCTCTAGAGCCATCCTCACCATC -3'. All constructs were verified by DNA sequencing. AEB071 , AHT956 , MEK162 and INC280 were synthesized at Novartis Pharma AG (East Hanover, NJ, USA). Human HGF is from Peprotech(Rocky Hill, NJ). PD0325901 and Trametinib were obtained from Chemie TEK(Indianapolis, IN). TPA and Verteporfin were purchased from Sigma (St Louis, MO)

Cell culture

The sources of uveal melanoma cell lines have been previously described(Griewank et al., 2012). Cutaneous melanoma cell lines SK-MEL-5, UACC257, MM415, MM485, SK-MEL-28 were purchased from UCSF cell culture facility. All melanoma cell lines were maintained in RPMI 1640 with 10%FBS. Human Burkitt's lymphoma Ramos cells were obtained from ATCC (Manassas, VA) and were maintained in RPMI 1640 with 10%FBS. 293FT cells were obtained from Invitrogen (Grand Island, NY) and were cultured in DMEM with 10%FBS. Immortalized human melanocytes (a gift from Dr. David Fisher, Dana Farber Cancer Institute, Boston, MA) and normal human melanocytes from Invitrogen were cultured in 254 media supplemented by human melanocyte growth supplements (HMGS-2; Invitrogen). Melan-a cells (a gift from Dr Dorothy Bennett, St George University, London, UK) were maintained in RPMI with 10% FBS and 200 nM TPA TPA .

Transient transfection, lentiviral transduction and siRNA mediated gene knockdown

For transient transfection, 293FT cells were transfected using lipofectamine 2000(Invitrogen) following the manufacturer's instruction. Cells were processed 24h-48h after transfection. Lentiviral generation and transduction have been described previously (Chen et al., 2014). All siRNA pools were obtained from Dharmacon (Rockford, IL). The list of siRNA for different genes is shown in the supplementary procedures. 30nM siRNAs for each gene were transfected using lipofectamine RNAimax reagent according to manufacturer's instructions (Invitrogen). For combination knock down, non-target siRNAs were used to keep each sample same amount of total siRNAs. After 48 to 72h transfection, cells were either lysed or used for cell proliferation analysis.

Western blot analysis and cell proliferation

Cell lysis and immunoblotting were performed as described(Chen et al., 2014). For antibody details, see supplementary information. Western blot bands were subjected to densitometric analysis using image Studio software from Li-COR Biosciences (Lincoln, Nebraska). For short term proliferation assays (over one week), cells were transfected

with indicated siRNAs. After 48h transfection, cells were counted using TC10 automated cell counter(Bio-Rad, Hercules, CA) and plated in triplicate into 6-well plates at $5x10^4$ cells/well and cultured for the indicated times. Cells were collected by trypsinization and counted in a TC10 automated cell counter (Bio-Rad, Hercules, CA). For long term proliferation assays, cells were plated at 10 cm plates and then treated with or without inhibitors. After 12 to 18 days, cells were stained with Crystal Violet.

Assay for activated Ras

The Ras activation assay was performed using an agarose-bound GST-fused RBD of Raf-1 as described by the manufacturer (Millipore). Briefly, 293FT cells were transfected with indicated plasmids for 24h and then were washed with cold PBS and lysed. The activated Ras was pulled down with agarose-conjugated Raf-1 RBD, followed by SDS/PAGE gel electrophoresis and immunoblotting with a pan-Ras antibody or a Ras isoform-specific antibody.

Taqman Real time RT-PCR

RNA was isolated from cells by using RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacture's instruction and reverse-transcribed with random primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase. Real-time PCR was performed in duplicate or triplicate using Eppendorf RealPlex2. Gene expression was evaluated using SensiMix II probe (Bioline) and specific probes described below. Each sample has been normalized to GAPDH and quantified with the comparative CT method according to the manufacturer's instructions. The following combinations of primers and probes were used to analyze the expression of human RasGRP1: forward, AAGCTCCACCAACTACAGAACT; reverse, AGGGAGATGAGGTCCTTGAGAT; probe, FAM-CCACATGAAATCAATAAGGTTCTCGGTGAG-TAMRA and human GAPDH forward, GAAGGTGAAGGTCGGAGT; reverse, GAAGATGGTGATGGGATTTC; probe, FAM-AGGCTGAGAACGGGAAGCTTGT-TAMRA. SOS1 (Hs00362308_m1), RasGRP2 (Hs00183378_m1), 3 (Hs00209808_m1) and 4 (Hs01073182_m1) probes and primers were obtained at Applied Bio System.

Microarray experiments

Purified RNA was analyzed for quality using chip-based capillary electrophoresis (Bioanalyzer, Agilent, Inc) and quantity and purity was determined with a NanoDrop spectrometer. The GeneChip WT PLUS Reagent kit was used for amplification, fragmentation and biotin-labeling (Affymetrix, Santa Clara, CA). The labeled cDNA was hybridized to Human Transcriptome 2.0 microarrays (Affymetrix, Santa Clara, CA). The signal intensity fluorescent images produced during Affymetrix GeneChip hybridizations were read using the Affymetrix Model 3000 Scanner and converted into GeneChip probe results files (CEL) using Command and Expression Console software (Affymetrix). The array data were normalized at gene level and outliers were identified and removed if have by using Affymetrix expression console software. Gene level differential expression was compared between 5 GNAQ/11 mutant melanoma cells and 5 BRAF/Nras mutant melanoma cells by using Affymetrix transcriptome Analysis Console (TAC) software. Algorithm option is one-way between-subject Anova (Unpaired). Filter criteria: (1) fold change (linear)<-2 or fold change (linear)>2; (2) Anova p-value <0.05. Human Transcriptome Array 2.0 covered more than 285,000 full-length transcripts including >245,000coding transcripts. Total 67528 genes are coved on this array including 44699 coding genes.

Human uveal melanoma cell lines and human uveal melanoma tissues Immunohistochemistry

The 92.1, Omm1 and Mel270 UM cell lines were grown in RPMI (Life Technologies, Paisley, UK) plus 10% FCS. The CRMM1 and CRMM2 conjunctival melanoma cell lines were grown in Ham's F-12K (Kaighn's) Medium (Life Technologies) plus 10% FCS. At ~75% confluence the cells were removed from the bottom of a 75cm^2 tissue culture flask using a cell scraper. The cells were then pelleted at 1500rpm for 2 minutes and fixed in 10% neutral buffered formalin for 10 minutes. Following fixation the cells were washed in PBS before transferring the cell pellet to a microfuge tube for a final wash with PBS and centrifugation at 8000rpm for 1 minute. The supernatant was removed and 100µl of 4% molten agar was added and mixed quickly into the cell pellet prior to centrifugation at 14,000rpm for 30 seconds. The agar cell pellet was then removed from

the microfuge tube and placed into a tissue cassette for processing through a series of alcohols and xylenes. After dehydration the pellet was paraffin wax embedded such that the agar tip containing cells was at the surface of the block.

Sections (4 μm thick) cut from the cell pellet blocks, human uveal melanoma specimens underwent immunohistochemistry as previously described (Lake et al., 2013). In brief, antigen retrieval and IHC were performed using the Dako PT Link and Autostainer Plus systems and Envision™ FLEX Kit according to standard manufacturers' procedures (Dako UK Ltd, Cambridgeshire, UK). Positive staining was visualized either by use of 3-amino-9-ethylcarbazole (AEC, Vector Laboratories Ltd., Peterborough, UK, 30 min). The slides were counterstained with Mayer's hematoxylin (VWR, Leicestershire, UK) and cover-slipped using an aqueous mountant (Aquatex™, VWR). The following RasGRP3 primary antibody was used: rabbit polyclonal, 13162-1-AP (ProteinTech, Manchester, UK) at 1:200. Omission of the primary antibody served as the negative control for the rabbit polyclonal. Human duodenal tissue served as a positive control.

Mouse xenograft

The constructs containing shRNA sequence targeting human RasGRP3 or non-target shRNAs were obtained from Genecopoeia (Rockville, MD). The RasGRP3 shRNA expressing stable 92-1 cell lines were generated following the manufacturer's instructions. Nude mice (Jackson laboratory) were injected subcutaneously in the flanks with $2x10^6$ cells suspended in matrigel. Mice were monitored for 13 weeks after injection and then were sacrificed. Tumors were collected and weighted. All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Our research was supported by an R01 grant CA142873 from the National Cancer Institute and a Stein Innovation Award from Research to Prevent Blindness (To BCB), a Young Investigator Award from the Melanoma Research Alliance (to XC), the Sandler Program in Basic Science (start-up), NIH-NCI Physical Science Oncology Center grant U54CA143874, NIH grant 1P01AI091580-01, a Gabrielle's Angel Foundation grants, a UCSF ACS grant, and a UCSF Research Allocation Program (RAP) pilot grant (all to JPR).

References

Aiba, Y., Oh-hora, M., Kiyonaka, S., Kimura, Y., Hijikata, A., Mori, Y., and Kurosaki, T. (2004). Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation. Proceedings of the National Academy of Sciences of the United States of America *101*, 16612-16617.
Bollag, G., Hirth, P., Tsai, J., Zhang, J., Ibrahim, P.N., Cho, H., Spevak, W., Zhang, C., Zhang, Y., Habets, G.*, et al.* (2010). Clinical efficacy of a RAF inhibitor needs broad target blockade in BRAF-mutant melanoma. Nature *467*, 596-599.
Chen, X., and Resh, M.D. (2001). Activation of mitogen-activated protein kinase by membrane-targeted Raf chimeras is independent of raft localization. The Journal of biological chemistry *276*, 34617-34623.
Chen, X., Wu, Q., Tan, L., Porter, D., Jager, M.J., Emery, C., and Bastian, B.C. (2014). Combined PKC and MEK inhibition in uveal melanoma with GNAQ and GNA11 mutations. Oncogene *33*, 4724-4734.
Della Rocca, G.J., van Biesen, T., Daaka, Y., Luttrell, D.K., Luttrell, L.M., and Lefkowitz, R.J. (1997). Ras-dependent mitogen-activated protein kinase activation by G protein-coupled receptors. Convergence of Gi- and Gq-mediated pathways on calcium/calmodulin, Pyk2, and Src kinase. The Journal of biological chemistry *272*, 19125-19132.
Denning, M.F. (2012). Specifying protein kinase C functions in melanoma. Pigment cell & melanoma research *25*, 466-476.
Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S.A., and Schlessinger, J. (1996). A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature *383*, 547-550.
Dissanayake, S.K., Wade, M., Johnson, C.E., O'Connell, M.P., Leotlela, P.D., French, A.D., Shah, K.V., Hewitt, K.J., Rosenthal, D.T., Indig, F.E.*, et al.* (2007). The Wnt5A/protein kinase C pathway mediates motility in melanoma cells via the inhibition of metastasis suppressors and initiation of an epithelial to mesenchymal transition. The Journal of biological chemistry *282*, 17259-17271.
Feng, X., Degese, M.S., Iglesias-Bartolome, R., Vaque, J.P., Molinolo, A.A., Rodrigues, M., Zaidi, M.R., Ksander, B.R., Merlino, G., Sodhi, A.*, et al.* (2014). Hippo-independent activation of YAP by the GNAQ uveal melanoma oncogene through a trio-regulated rho GTPase signaling circuitry. Cancer cell *25*, 831-845.
Flaherty, K.T., Infante, J.R., Daud, A., Gonzalez, R., Kefford, R.F., Sosman, J., Hamid, O., Schuchter, L., Cebon, J., Ibrahim, N.*, et al.* (2012). Combined BRAF and MEK Inhibition in Melanoma with BRAF V600 Mutations. The New England journal of medicine.
Gilhooly, E.M., Morse-Gaudio, M., Bianchi, L., Reinhart, L., Rose, D.P., Connolly, J.M., Reed, J.A., and Albino, A.P. (2001). Loss of expression of protein kinase C beta is a common phenomenon in human malignant melanoma: a result of transformation or differentiation? Melanoma research *11*, 355-369.
Griewank, K.G., Yu, X., Khalili, J., Sozen, M.M., Stempke-Hale, K., Bernatchez, C., Wardell, S., Bastian, B.C., and Woodman, S.E. (2012). Genetic and molecular characterization of uveal melanoma cell lines. Pigment cell & melanoma research *25*, 182-187.
Griner, E.M., and Kazanietz, M.G. (2007). Protein kinase C and other diacylglycerol effectors in cancer. Nature reviews Cancer *7*, 281-294.

Guo, F.F., Kumahara, E., and Saffen, D. (2001). A CalDAG-GEFI/Rap1/B-Raf cassette couples M(1) muscarinic acetylcholine receptors to the activation of ERK1/2. The Journal of biological chemistry *276*, 25568-25581.
Hubbard, K.B., and Hepler, J.R. (2006). Cell signalling diversity of the Gqalpha family of heterotrimeric G proteins. Cellular signalling *18*, 135-150.
Johansson, P., Aoude, L.G., Wadt, K., Glasson, W.J., Warrier, S.K., Hewitt, A.W., Kiilgaard, J.F., Heegaard, S., Isaacs, T., Franchina, M.*, et al.* (2016). Deep sequencing of uveal melanoma identifies a recurrent mutation in PLCB4. Oncotarget *7*, 4624-4631.
Khalili, J.S., Yu, X., Wang, J., Hayes, B.C., Davies, M.A., Lizee, G., Esmaeli, B., and Woodman, S.E. (2012). Combination small molecule MEK and PI3K inhibition enhances uveal melanoma cell death in a mutant GNAQ- and GNA11-dependent manner. Clinical cancer research : an official journal of the American Association for Cancer Research *18*, 4345-4355.
Kolch, W., Heidecker, G., Kochs, G., Hummel, R., Vahidi, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U.R. (1993). Protein kinase C alpha activates RAF-1 by direct phosphorylation. Nature *364*, 249-252.
Ksionda, O., Limnander, A., and Roose, J.P. (2013). RasGRP Ras guanine nucleotide exchange factors in cancer. Frontiers in biology *8*, 508-532.
Lake, S.L., Damato, B.E., Kalirai, H., Dodson, A.R., Taktak, A.F., Lloyd, B.H., and Coupland, S.E. (2013). Single nucleotide polymorphism array analysis of uveal melanomas reveals that amplification of CNKSR3 is correlated with improved patient survival. The American journal of pathology *182*, 678-687.
Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J.M., Plowman, G.D., Rudy, B., and Schlessinger, J. (1995). Protein tyrosine kinase PYK2 involved in Ca(2+)-induced regulation of ion channel and MAP kinase functions. Nature *376*, 737-745.
Lorenzo, P.S., Kung, J.W., Bottorff, D.A., Garfield, S.H., Stone, J.C., and Blumberg, P.M. (2001). Phorbol esters modulate the Ras exchange factor RasGRP3. Cancer research *61*, 943-949.
Moore, A.R., Ceraudo, E., Sher, J.J., Guan, Y., Shoushtari, A.N., Chang, M.T., Zhang, J.Q., Walczak, E.G., Kazmi, M.A., Taylor, B.S.*, et al.* (2016). Recurrent activating mutations of G-protein-coupled receptor CYSLTR2 in uveal melanoma. Nature genetics *48*, 675-680.
Nagy, Z., Kovacs, I., Torok, M., Toth, D., Vereb, G., Buzas, K., Juhasz, I., Blumberg, P.M., Biro, T., and Czifra, G. (2014). Function of RasGRP3 in the formation and progression of human breast cancer. Molecular cancer *13*, 96.
Oh-hora, M., Johmura, S., Hashimoto, A., Hikida, M., and Kurosaki, T. (2003). Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor signaling. The Journal of experimental medicine *198*, 1841-1851.
Oka, M., and Kikkawa, U. (2005). Protein kinase C in melanoma. Cancer metastasis reviews *24*, 287-300.
Schonwasser, D.C., Marais, R.M., Marshall, C.J., and Parker, P.J. (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Molecular and cellular biology *18*, 790-798.
Selzer, E., Okamoto, I., Lucas, T., Kodym, R., Pehamberger, H., and Jansen, B. (2002). Protein kinase C isoforms in normal and transformed cells of the melanocytic lineage. Melanoma research *12*, 201-209.

Sheldahl, L.C., Park, M., Malbon, C.C., and Moon, R.T. (1999). Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Current biology : CB *9*, 695-698.
Shin, M.K., Levorse, J.M., Ingram, R.S., and Tilghman, S.M. (1999). The temporal requirement for endothelin receptor-B signalling during neural crest development. Nature *402*, 496-501.
Singh, A.D., Bergman, L., and Seregard, S. (2005). Uveal melanoma: epidemiologic aspects. Ophthalmology clinics of North America *18*, 75-84, viii.
Soh, J.W., and Weinstein, I.B. (2003). Roles of specific isoforms of protein kinase C in the transcriptional control of cyclin D1 and related genes. The Journal of biological chemistry *278*, 34709-34716.
Teixeira, C., Stang, S.L., Zheng, Y., Beswick, N.S., and Stone, J.C. (2003). Integration of DAG signaling systems mediated by PKC-dependent phosphorylation of RasGRP3. Blood *102*, 1414-1420.
Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K., and Ohno, S. (1996). Protein kinase C activates the MEK-ERK pathway in a manner independent of Ras and dependent on Raf. The Journal of biological chemistry *271*, 23512-23519.
Van Raamsdonk, C.D., Bezrookove, V., Green, G., Bauer, J., Gaugler, L., O'Brien, J.M., Simpson, E.M., Barsh, G.S., and Bastian, B.C. (2009). Frequent somatic mutations of GNAQ in uveal melanoma and blue naevi. Nature *457*, 599-602.
Van Raamsdonk, C.D., Griewank, K.G., Crosby, M.B., Garrido, M.C., Vemula, S., Wiesner, T., Obenauf, A.C., Wackernagel, W., Green, G., Bouvier, N.*, et al.* (2010). Mutations in GNA11 in uveal melanoma. The New England journal of medicine *363*, 2191-2199.
Wu, X., Li, J., Zhu, M., Fletcher, J.A., and Hodi, F.S. (2012). Protein kinase C inhibitor AEB071 targets ocular melanoma harboring GNAQ mutations via effects on the PKC/Erk1/2 and PKC/NF-kappaB pathways. Molecular cancer therapeutics *11*, 1905-1914.
Yang, D., Kedei, N., Li, L., Tao, J., Velasquez, J.F., Michalowski, A.M., Toth, B.I., Marincsak, R., Varga, A., Biro, T.*, et al.* (2010). RasGRP3 contributes to formation and maintenance of the prostate cancer phenotype. Cancer research *70*, 7905-7917.
Yang, D., Tao, J., Li, L., Kedei, N., Toth, Z.E., Czap, A., Velasquez, J.F., Mihova, D., Michalowski, A.M., Yuspa, S.H.*, et al.* (2011). RasGRP3, a Ras activator, contributes to signaling and the tumorigenic phenotype in human melanoma. Oncogene *30*, 4590-4600.
Yu, F.X., Luo, J., Mo, J.S., Liu, G., Kim, Y.C., Meng, Z., Zhao, L., Peyman, G., Ouyang, H., Jiang, W.*, et al.* (2014). Mutant Gq/11 promote uveal melanoma tumorigenesis by activating YAP. Cancer cell *25*, 822-830.
Zheng, Y., Liu, H., Coughlin, J., Zheng, J., Li, L., and Stone, J.C. (2005). Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. Blood *105*, 3648-3654.

Figure legends

Figure 1 PKC δ and PKC ε are important downstream effectors of oncogenic GNAQ in mediating MAPK signaling. A) Expression of PKC isoforms in melanoma cell lines with and without GNAQ or GNA11 mutations. Previously validated antibodies against various PKC isoforms (Fig.S1A and S1B) were tested on a broad panel of melanoma cell lines. Suitable positive controls (PC) for isoforms found not to be expressed are shown on the right most lane. **B)** Knockdown of GNAQ and PKC isoforms δ and ε reduced levels of pMEK and pERK in the GNAQ mutated uveal melanoma cell line 92-1, but not in the GNAQ wild type cell line C8161 (C). Cells were transfected with respective siRNAs either singly or in combination and then lysed. Abbreviations: -: no siRNA ; NT: non-targeting siRNA. **D)** PKC δ and PKC ε synergized with $GNAQ^{Q209L}$ in activating MAPK in 293FT cells. 293FT cells were transfected for 24h. PKC isoforms expression was detected using HA tags and $GNAQ^{Q209L}$ using a Glu-Glu tag (EE). **E)** Effect of knockdown of PKC α, PKC δ, PKC ε and GNAQ or GNA11 on the proliferation of GNAQ mutant melanoma cells (92-1 and OMM1.3) and GNA11 mutant cell line (UPMD-2) and GNAQ/11 wild type melanoma cells (C8161 and SK-MEL-28 from cutaneous origin and MEL285 from uveal origin). Cells were transfected with various combination of siRNAs as indicated. 48h after transfection, cells were counted and seeded at equal density. Cells were counted at day 1, 3, 5 after plating. Abbreviations: -: no siRNA control . NT: non-targeting siRNA control.

Figure 2 Oncogenic GNAQ activates Ras A) $GNAQ^{Q209L}$ transfected alone or combined with PKC δ or PKC ε into 293FT cells increased Ras-GTP levels. 293FT cells

were transfected with indicated cDNA plasmids for 24h. Ras-GTP pull-down was performed and detected by Western blot with a pan-Ras antibody. TPA stimulation was used a positive control. Expression levels of $GNAQ^{Q209L}$ were monitored with Glu-Glu (EE) tag. PKC δ and PKC ε were detected via HA tags. **B)** Oncogenic GNAQ activates all three Ras isoforms. 293FT cells were transfected with indicated cDNA plasmids for 24h. Ras-GTP pull-down was performed and detected by Western blot with Ras isoform-specific antibodies. **C)** Depletion of Ras isoforms reduced MAPK signaling as evidenced by decreased pMEK and pERK in GNAQ mutant melanoma cell lines. Three human uveal melanoma cell lines with GNAQ mutation (92-1, OMM1.3 and MEL202) were transfected with mock (-), non-targeting siRNAs (NT), siRNAs against GNAQ, Hras(H), Kras(K), Nras(N) or indicated combinations for 72h and lysed.

Figure 3 RasGRP3 is upregulated in uveal melanoma cells with GNAQ/11 mutations. A) Scatter plot of top 487 genes (left panel) and heatmap of top 30 genes(right panel) of differentially expressed genes between 5 GNAQ/11 mutant (mt) and 5 GNAQ/11 wild type (wt). **B)** Quantitative RT-PCR shows markedly elevated RasGRP3 mRNA levels in 6 GNAQ mutant cells (92-1, MEL202, OMM1.3, MEL270, UPMM2 and UPMM3) and 3 GNA11 mutant cells (OMM-GN11, UPMD-1 and UPMD-2), compared to 3 types of human melanocytes (IHM: immortalized human melanocytes; NHM: normal human melanocytes) and 7 human melanoma cell lines without GNAQ/11 mutations. Three (MUM2C, MEL285 and MEL290) melanoma cell lines without GNAQ/11 mutations are from uveal origin. **C)** RasGRP3 mRNA expression across different human cancers from TCGA RNAseq data. RasGRP3 expression is highest in

uveal melanoma. **D)** Cutaneous melanomas with $GNAQ^{Q209P}$ or $GNA11^{Q209L}$ mutations in the TCGA have increased RasGRP3 mRNA expression, comparable to uveal melanomas. E) Western blot shows increased RasGRP3 protein levels in human melanoma cells with GNAQ/11 mutations compared to cell lines with other mutations (MEL285, MUM2C and MEL290 are of uveal and the other GNAQ/11 wt cell lines are of cutaneous origin). RasGRP1, RasGRP2 and RasGRP4 were not detectable in melanoma cells with GNAQ/11 mutations. 293FT lysates transfected with RasGRPs cDNA were used as positive control. F) RasGRP3 immunohistochemistry in human uveal melanoma cell lines. RasGRP3 immunohistochemistry of pellets of formalin-fixed paraffin-embedded GNAQ mutant UM cell lines (92-1 and MEL270) and GNAQ/11 wild type lines (CRMM1 and CRMM2). G) RasGRP3 immunohistochemistry in 4 uveal melanoma tissues with GNAQ mutation and 4 with GNA11 mutations.

Figure 4 Oncogenic GNAQ signaling regulates RasGRP3 expression and phosphorylation in uveal melanoma cells. A) Depletion of GNAQ with siRNAs decreased RasGRP3 expression and T133 phosphorylation in three GNAQ mutant uveal melanoma cells (OMM1.3, 92-1 and MEL202). Cells were transfected with mock (-), non-targeting siRNAs (NT), or GNAQ siRNAs for 72 h and lysed. **B)** $GNAQ^{Q209L}$ or $GNA11^{Q209L}$ but not wild-type GNAQ, GNA11 or $Braf^{V600E}$ induced increased RasGRP3 expression and phosphorylation in immortalized human melanocytes. Melanocytes were infected with lentivirus expressing indicated proteins for 48h and analyzed by immunoblot. Expression levels of GNAQ and GNA11 were monitored using Glu-Glu tags. **C)** The PKC inhibitor AEB071 decreased RasGRP3 expression and

phosphorylation in OMM1.3 cells in a time dependent manner. OMM1.3 cells were incubated with DMSO or 500nM AEB071 for the times indicated. **D)** The PKC inhibitors AEB071 and AHT956 decreased RasGRP3 expression and phosphorylation in OMM1.3 cells in a dose dependent manner. OMM1.3 cells were incubated with DMSO or AEB071 or AHT956 at indicated doses for 24h. **E)** TPA increased RasGRP3 expression and phosphorylation in human melanocytes. Immortalized human melanocytes were incubated with or without 200nM TPA for the indicated times. F) PKC δ and PKC ε increase T133 phosphorylation of RasGRP3 in 293FT cells. 293FT cells were transfected with indicated cDNAs for 24h. G**)** Knockdown of PKC δ and PKC ε decreased RasGRP3 expression and phosphorylation in GNAQ mutant cells. Western blot of 92-1 cells transfected for 72h with mock (-), non-targeting siRNAs (NT), PKC α, δ, or ε siRNAs, singly or in indicated combination. H) The MEK inhibitor PD0325901 has no effect on RasGRP3 expression and phosphorylation in GNAQ mutant cells. MEL202 and 92-1 cells were incubated with PD0325901 at different doses for 24h.

Figure 5 RasGRP3 is important for GNAQ-mediated MAP-kinase pathway activation and proliferation. A) siRNA mediated knock down of RasGRP3 decreases pMEK and pERK levels in three GNAQ mutant melanoma cells (92-1, omm1.3 and mel270) but not in three GNAQ/11 wild type melanoma cells (B). MUM2C is from uveal origin. Cells were transfected with mock(-), non-targeting or RasGRP3 siRNAs for 72h and lysed for Western blot analysis. C) RasGRP3 knockdown reduces proliferation of melanoma cells with GNAQ mutations (92-1, MEL270 and OMM1.3) but not of cell lines with other mutations (SK-MEL-5 and UACC257). Cells were transfected with non-

targeting or RasGRP3 siRNAs. 48h after transfection, cells were counted and plated at equal density. Cells were counted at day 1, 3, 5 after plating. D) Western blot of stable knockdown of RasGRP3 using shRNA. 92-1 cells were infected with lentivirus expressing two different shRNAs for RasGRP3 and selected with puromycin. NT: non-targeting shRNA. E) shRNA-mediated knockdown of RasGRP3 in 92-1 cells (from panel D) decreased the number and size of colonies. Cells were stained with crystal violet after 18 days of culture. F) Nude mice were injected subcutaneously with $2x10^6$ 92-1 cells expressing RasGRP3 shRNA-1(n=5) or non-targeting shRNA (n=5). Tumors sizes and weights for both experimental arms 13 weeks after injection. . G) The effect of co-transfection of $GNAQ^{Q209L}$ with 4 different RasGRP isoforms on MAPK signaling. 293FT cells were transfected with mock (-), GFP, $GNAQ^{Q209L}$, RasGRP1, RasGRP2, RasGRP3 or RasGRP4 plasmids either singly or in combination as indicated. After 24h, cells were lysed and blotted. Arrows indicate that pRasGRP1 antibodies recognized both RasGRP1 T184 phosphorylation and RasGRP3 T133 phosphorylation.

Figure 6 Both PKC mediated phosphorylation and C1 domain are required for RasGRP3-mediated MAPK activation. A) Mutating the PKC phosphorylation site of RasGRP3 (T133A) partially attenuated RasGRP3 mediated MAPK signaling in 293FT cells. 293FT cells were transfected with mock(-), GFP, $GNAQ^{Q209L}$, RasGRP3 ^{wt}($GRP3^{wt}$), RasGRP3^{T133A}($GRP3^{T133A}$) either singly or in indicated combination. After 24h, cells were lysed and subjected to Western blot. RasGRP3 wild type and mutant expression were detected with Myc tag antibody. GLU-GLU tag (EE) was used to detect $GNAQ^{Q209L}$. The top panel shows a schematic diagram of the RasGRP3 T133A

construct. **B)** PKC inhibition fails to completely abrogate RasGRP3-mediated MAPK signaling. 293FT cells were transfected with GFP, $GNAQ^{Q209L}$, $RasGRP3^{wt}$, or $RasGRP3^{T133A}$ either singly or in indicated combinations. After 24 h, transfected cells were treated with 1μM AEB071 (AEB) or vehicle for another 24h and then lysed. **C)** The MEK inhibitor PD0325901(PD) completely suppressed RasGRP3-mediated MAPK signaling. 293 FT cells were co-transfected with $GNAQ^{Q209L}$ and $RasGRP3^{T133A}$ or with $GNAQ^{Q209L}$ and $RasGRP3^{wt}$. After 24h, cells were treated for another 24h with DMSO, 1μM AEB071(AEB), 1μm AHT956 (AHT), 100nM PD0325901(PD), 1μM AEB+100nM PD or 1μM AHT956+100nM PD, respectively. **D)** Both T133 phosphorylation and C1 domain of RasGRP3 contribute to oncogenic GNAQ-mediated MAPK signaling. 293 FT cells were transfected with indicated constructs for 24h and lysed. Left panel shows that schematic diagram of the RasGRP3 C1 domain deletion constructs used. E) Restoration of MAPK activation by expression of membrane-targeted forms of RasGRP3 in 293FT cells. RasGRP3 constructs tagged with the membrane targeting motifs of Fyn and Src protein as shown in left panel. 293FT cells transfected with indicated RasGRP3 mutant constructs for 24h.

Fig.7 Model of oncogenic GNAQ activates MAPK signaling in uveal melanoma. GTP-bound GNAQ directly activates PLC β, which generates the second messenger DAG. DAG activates PKC δ and PKC ε by binding their C1 domains. The Ras activator RasGRP3 represents a critical signaling node linking oncogenic GNAQ to the RAS/RAF/MEK/ERK signaling pathway by three independent mechanisms: binding of

membrane recruitment by DAG, phosphorylation and upregulation of expression by PKC δ and PKC ε.

Fig.1

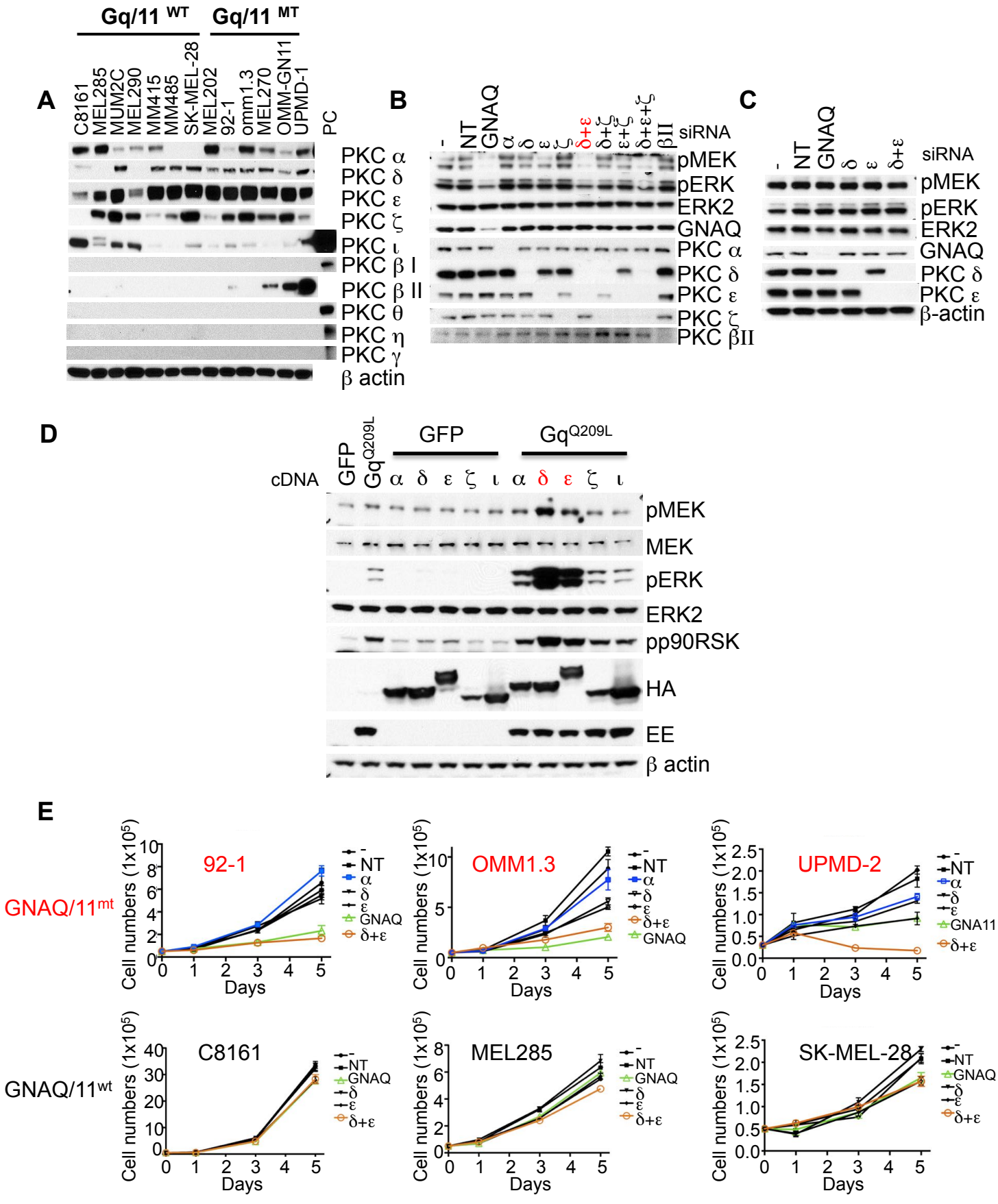

A
Gq/11 WT
Gq/11 MT
C8161
MEL285
MUM2C
MEL290
MM415
MM485
SK-MEL-28
MEL202
92-1
omm1.3
MEL270
OMM-GN11
UPMD-1
PC
PKC α
PKC δ
PKC ε
PKC ζ
PKC ι
PKC β I
PKC β II
PKC θ
PKC η
PKC γ
β actin
B
siRNA
-
NT
GNAQ
α
δ
ε
ζ
δ+ε
δ+ζ
ε+ζ
δ+ε+ζ
βII
pMEK
pERK
ERK2
GNAQ
PKC α
PKC δ
PKC ε
PKC ζ
PKC βII
C
siRNA
-
NT
GNAQ
δ
ε
δ+ε
pMEK
pERK
ERK2
GNAQ
PKC δ
PKC ε
β-actin
D
cDNA
GFP
GqQ209L
GFP
GqQ209L
α δ ε ζ ι α δ ε ζ ι
pMEK
MEK
pERK
ERK2
pp90RSK
HA
EE
β actin
E
GNAQ/11mt
GNAQ/11wt
92-1
OMM1.3
UPMD-2
C8161
MEL285
SK-MEL-28
Cell numbers (1x10^5)
Days
-
NT
α
δ
ε
ζ
GNAQ
GNA11
δ+ε

Fig.2

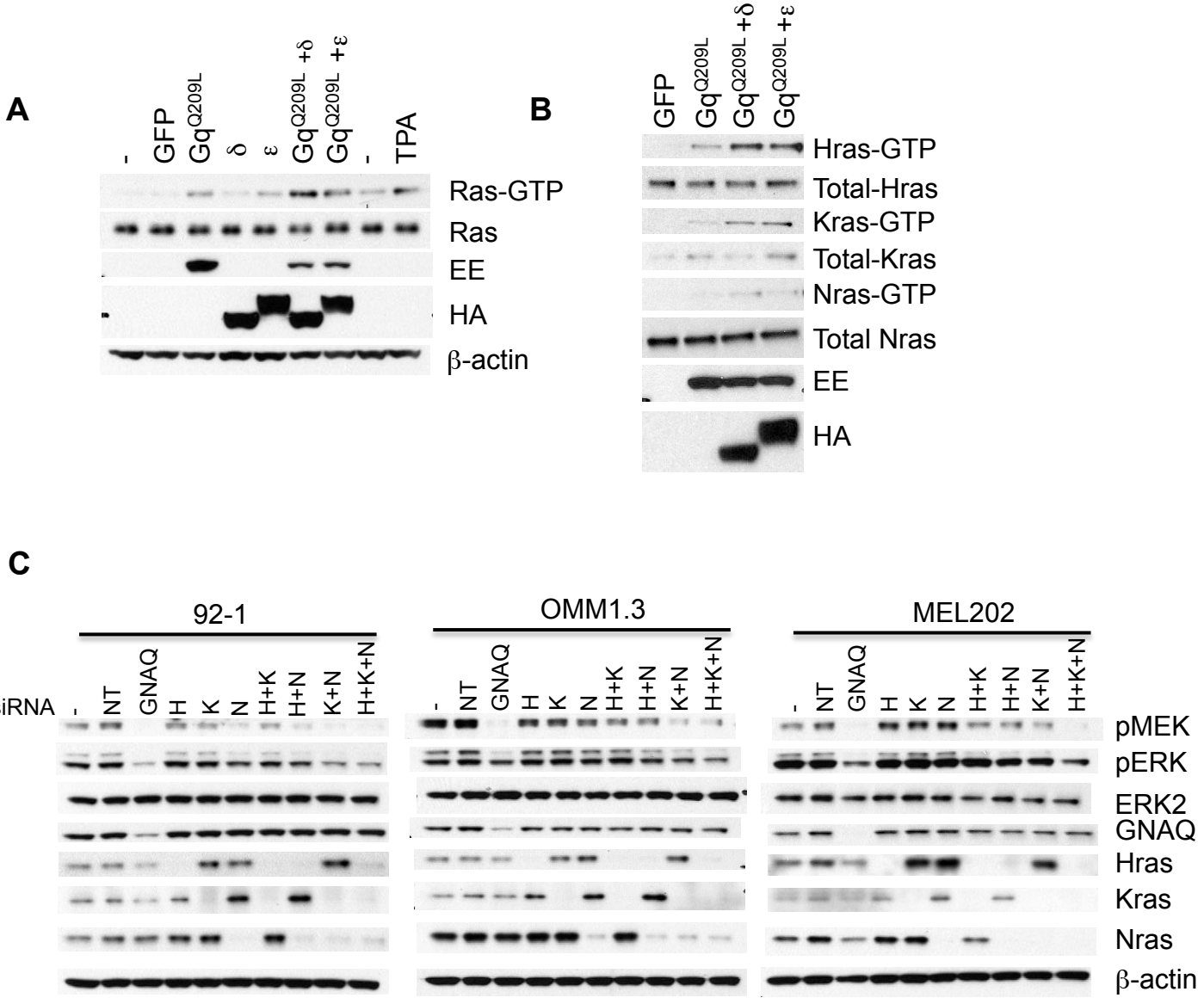

A
-
GFP
GqQ209L
δ
ε
GqQ209L +δ
GqQ209L +ε
-
TPA
Ras-GTP
Ras
EE
HA
β-actin
B
GFP
GqQ209L
GqQ209L +δ
GqQ209L +ε
Hras-GTP
Total-Hras
Kras-GTP
Total-Kras
Nras-GTP
Total Nras
EE
HA
C
92-1
OMM1.3
MEL202
siRNA
-
NT
GNAQ
H
K
N
H+K
H+N
K+N
H+K+N
pMEK
pERK
ERK2
GNAQ
Hras
Kras
Nras
β-actin

FIG.3

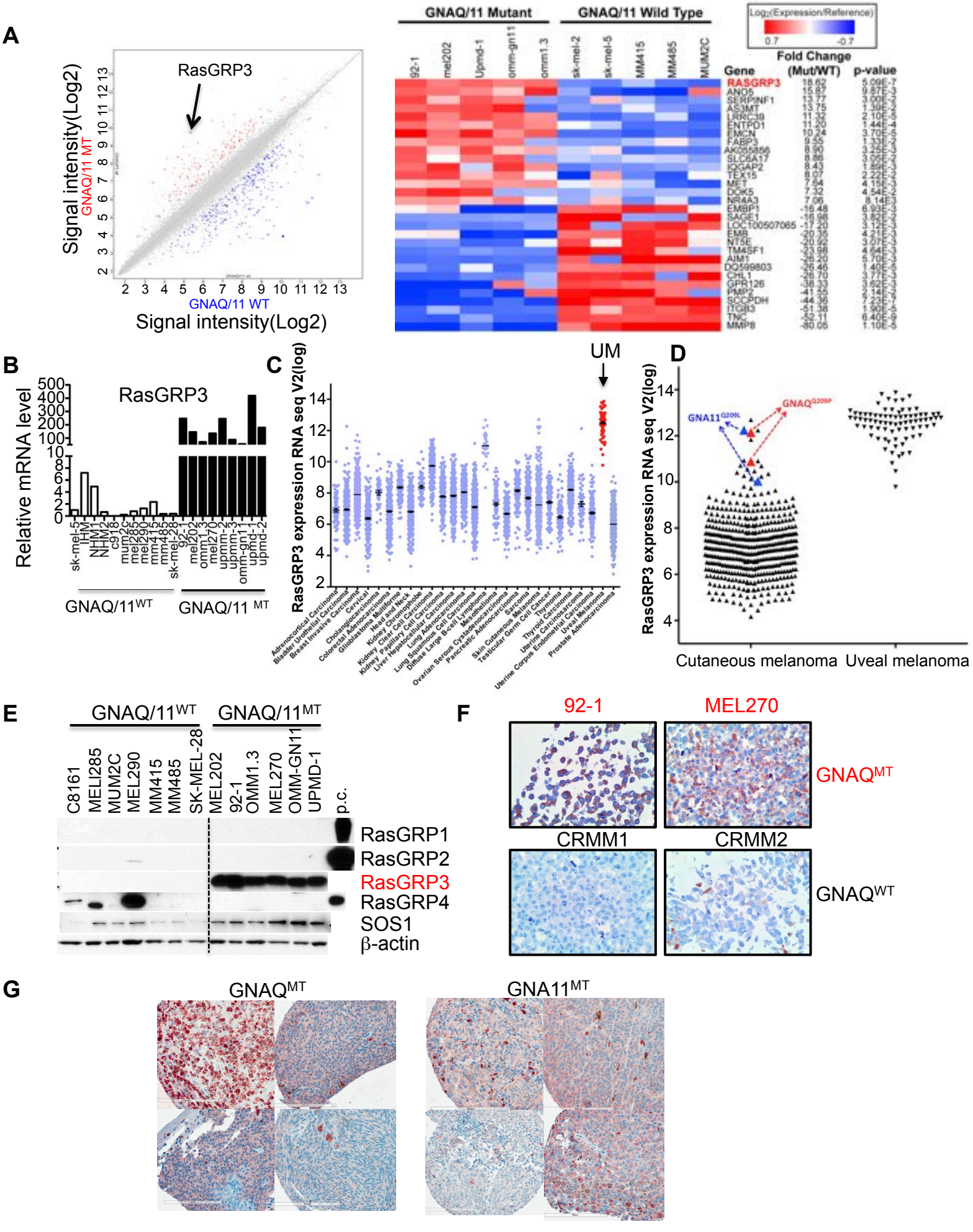

A
RasGRP3
Signal intensity(Log2)
GNAQ/11 MT
GNAQ/11 WT
Signal intensity(Log2)
GNAQ/11 Mutant
GNAQ/11 Wild Type
92-1
mel202
Upmd-1
omm-gn11
omm1.3
sk-mel-2
sk-mel-5
MM415
MM485
MUM2C
Log2(Expression/Reference)
0.7
-0.7
Fold Change
Gene
(Mut/WT)
p-value
RASGRP3
ANO5
SERPINF1
AS3MT
LRRC39
ENTPD1
EMCN
FABP3
SLC6A17
IQGAP2
TEX15
MET
DOK5
NR4A3
EMBP1
SAGE1
LOC100507065
EMB
NT5E
TM4SF1
AIM1
DQ599803
CHL1
GPR126
PMP2
SCCPDH
ITGB3
TNC
MMP8
B
RasGRP3
Relative mRNA level
sk-mel-5
IHM
NHM1
NHM2
c918
mum2c
mel285
mel290
mm415
mm485
sk-mel-28
92-1
mel202
omm1.3
mel270
upmm-2
upmm-3
omm-gn11
upmd-1
upmd-2
GNAQ/11WT
GNAQ/11 MT
C
UM
RasGRP3 expression RNA seq V2(log)
Adrenocortical Carcinoma
Bladder Urothelial Carcinoma
Breast Invasive Carcinoma
Cervical
Cholangiocarcinoma
Colorectal Adenocarcinoma
Glioblastoma Multiforme
Head and Neck
Kidney Chromophobe
Kidney Clear Cell Carcinoma
Kidney Papillary Cell Carcinoma
Liver Hepatocellular Carcinoma
Lung Adenocarcinoma
Lung Squamous Cell Carcinoma
Diffuse Large B-cell Lymphoma
Ovarian Serous Cystadenocarcinoma
Mesothelioma
Pancreatic Adenocarcinoma
Pheochromocytoma
Sarcoma
Skin Cutaneous Melanoma
Testicular Germ Cell Cancer
Thymoma
Thyroid Carcinoma
Uterine Carcinosarcoma
Uterine Corpus Endometrial Carcinoma
Uveal Melanoma
Prostate Adenocarcinoma
D
RasGRP3 expression RNA seq V2(log)
GNA11Q209L
GNAQQ209P
Cutaneous melanoma
Uveal melanoma
E
GNAQ/11WT
GNAQ/11MT
C8161
MELI285
MUM2C
MEL290
MM415
MM485
SK-MEL-28
MEL202
92-1
OMM1.3
MEL270
OMM-GN11
UPMD-1
p.c.
RasGRP1
RasGRP2
RasGRP3
RasGRP4
SOS1
β-actin
F
92-1
MEL270
GNAQMT
CRMM1
CRMM2
GNAQWT
G
GNAQMT
GNA11MT

Fig.4

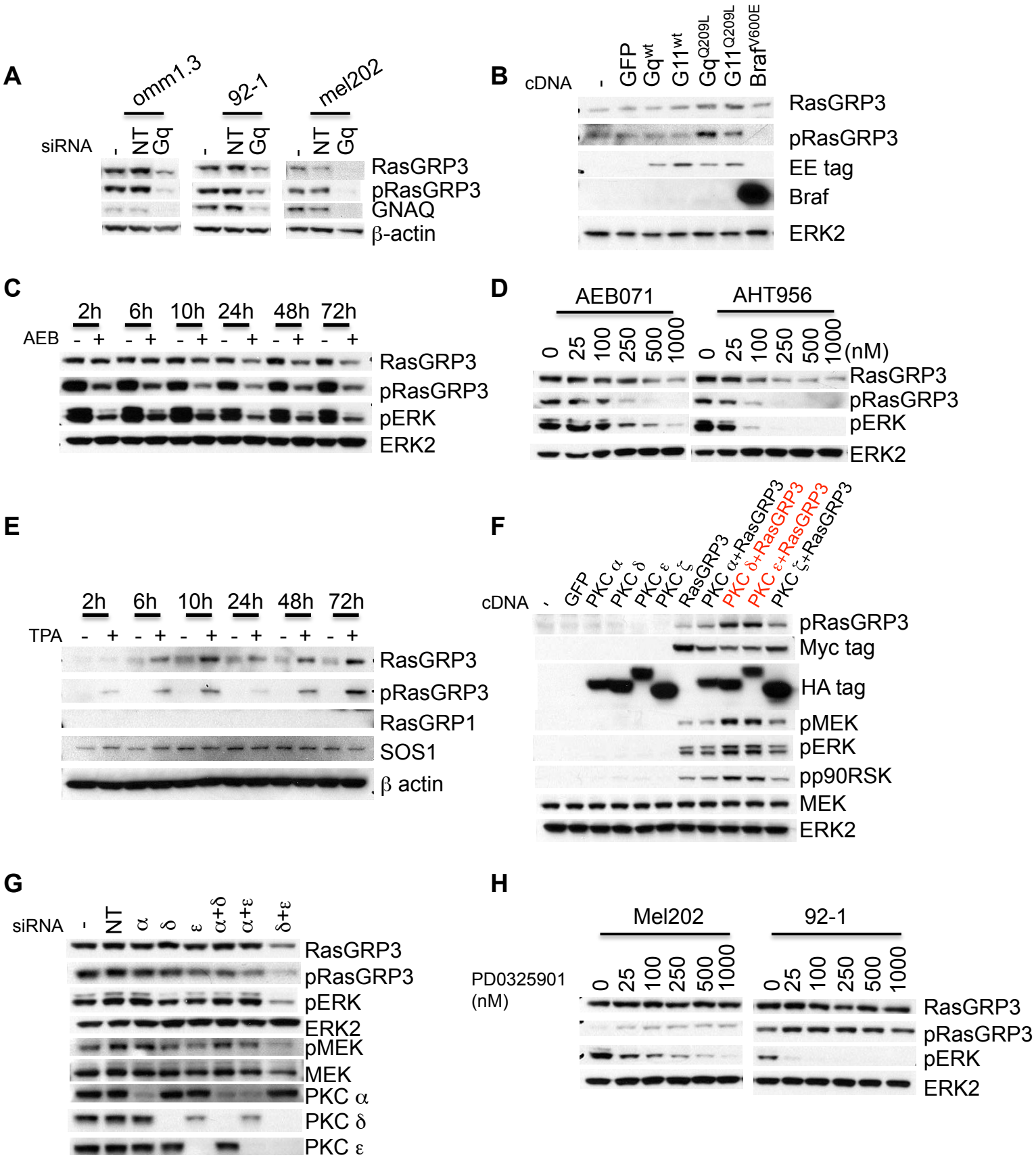

A
omm1.3
92-1
mel202
siRNA
-
NT
Gq
RasGRP3
pRasGRP3
GNAQ
β-actin
B
cDNA
-
GFP
Gq^wt
G11^wt
Gq^Q209L
G11^Q209L
Braf^V600E
RasGRP3
pRasGRP3
EE tag
Braf
ERK2
C
2h
6h
10h
24h
48h
72h
AEB
-
+
RasGRP3
pRasGRP3
pERK
ERK2
D
AEB071
AHT956
0
25
100
250
500
1000
(nM)
RasGRP3
pRasGRP3
pERK
ERK2
E
2h
6h
10h
24h
48h
72h
TPA
-
+
RasGRP3
pRasGRP3
RasGRP1
SOS1
β actin
F
cDNA
-
GFP
PKC α
PKC δ
PKC ε
PKC ζ
RasGRP3
PKC α+RasGRP3
PKC δ+RasGRP3
PKC ε+RasGRP3
PKC ζ+RasGRP3
pRasGRP3
Myc tag
HA tag
pMEK
pERK
pp90RSK
MEK
ERK2
G
siRNA
-
NT
α
δ
ε
α+δ
α+ε
δ+ε
RasGRP3
pRasGRP3
pERK
ERK2
pMEK
MEK
PKC α
PKC δ
PKC ε
H
Mel202
92-1
PD0325901
(nM)
0
25
100
250
500
1000
RasGRP3
pRasGRP3
pERK
ERK2

Fig.5

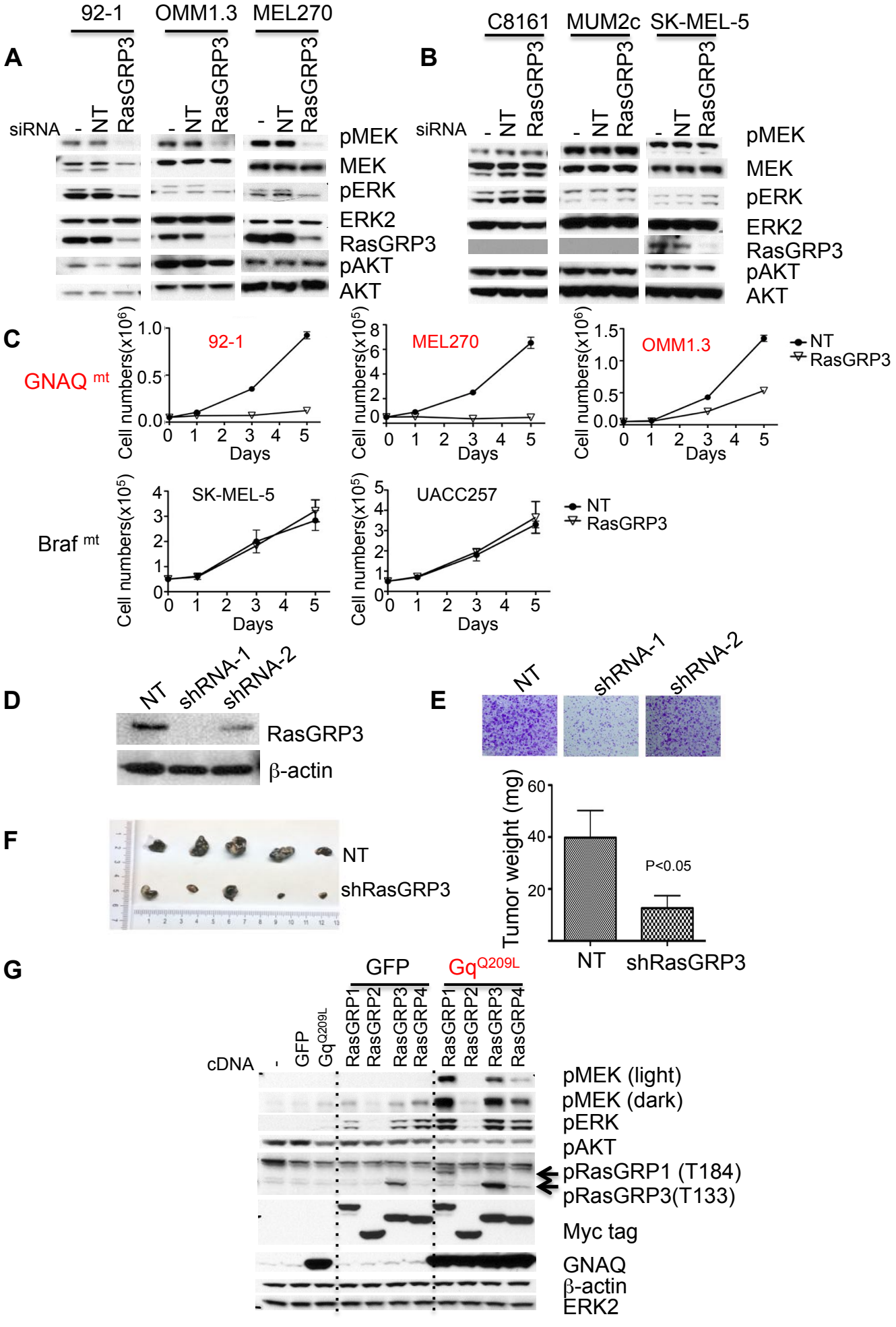

A
92-1
OMM1.3
MEL270
siRNA
-
NT
RasGRP3
pMEK
MEK
pERK
ERK2
RasGRP3
pAKT
AKT
B
C8161
MUM2c
SK-MEL-5
C
GNAQ mt
92-1
MEL270
OMM1.3
NT
RasGRP3
Cell numbers(x10^6)
Cell numbers(x10^5)
Days
Braf mt
SK-MEL-5
UACC257
D
NT
shRNA-1
shRNA-2
RasGRP3
β-actin
E
Tumor weight (mg)
P<0.05
F
NT
shRasGRP3
G
GFP
GqQ209L
RasGRP1
RasGRP2
RasGRP3
RasGRP4
cDNA
pMEK (light)
pMEK (dark)
pERK
pAKT
pRasGRP1 (T184)
pRasGRP3(T133)
Myc tag
GNAQ
β-actin
ERK2

Fig.6

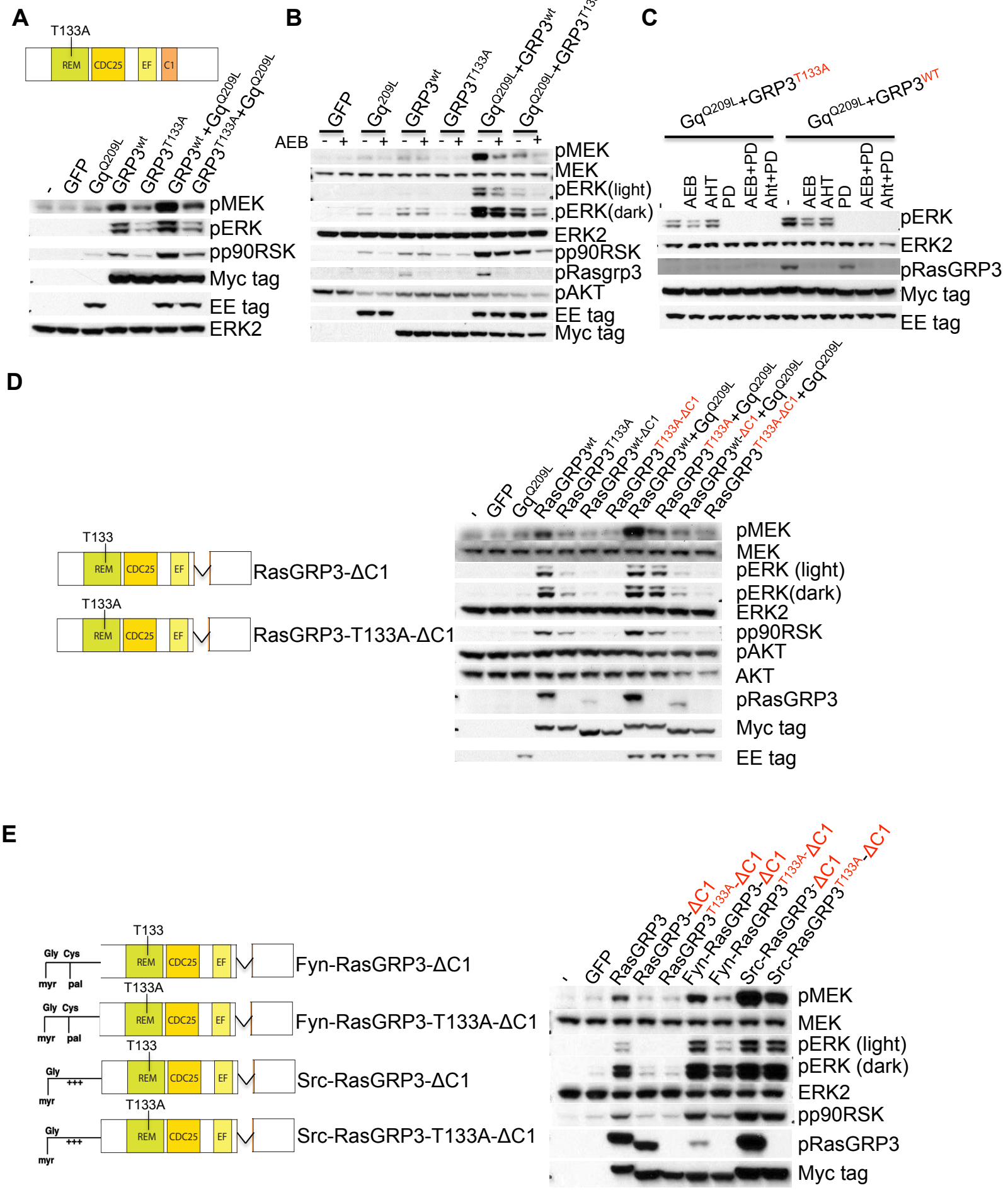
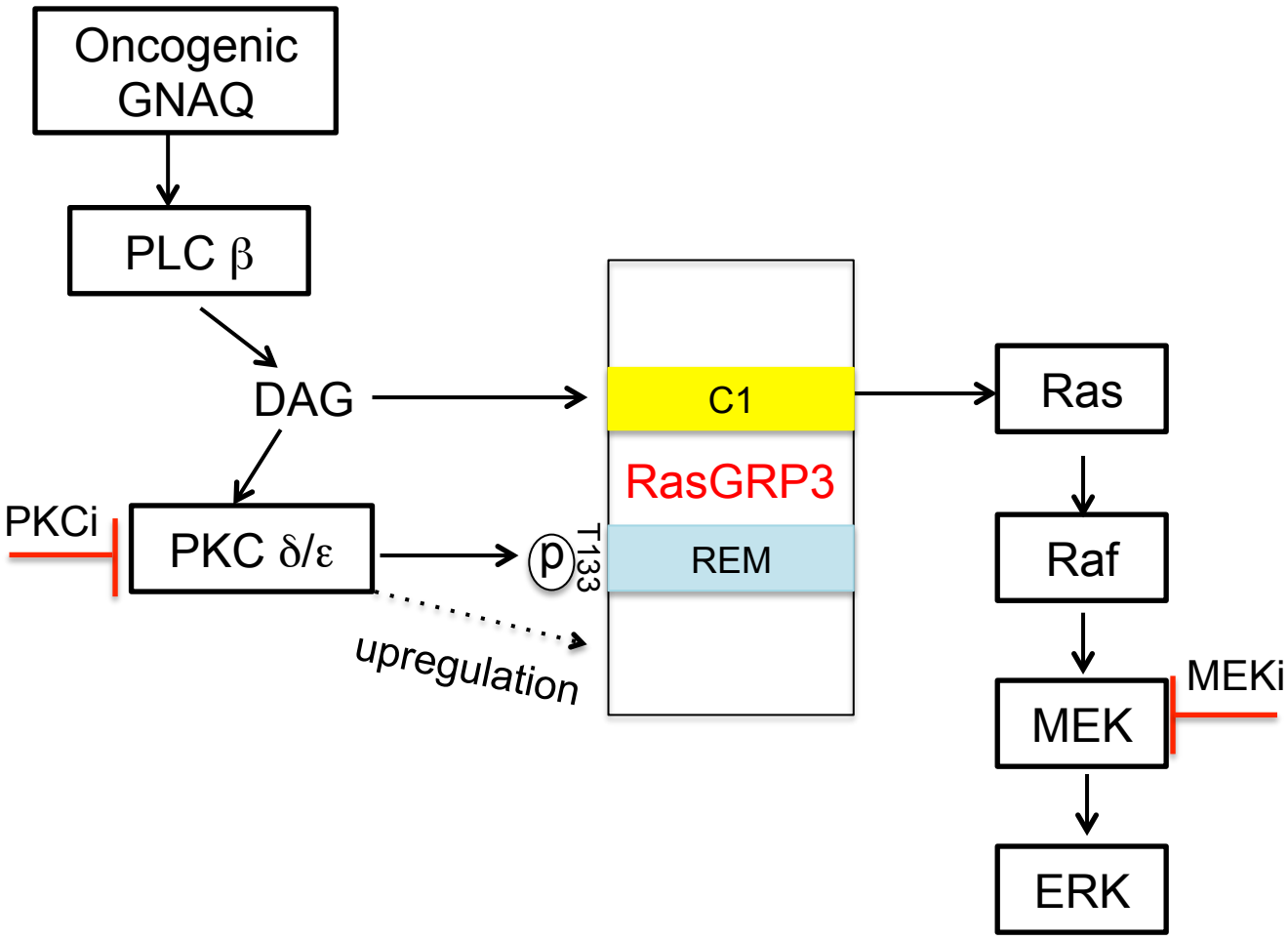

A
T133A
REM
CDC25
EF
C1
-
GFP
GqQ209L
GRP3wt
GRP3T133A
GRP3wt+GqQ209L
GRP3T133A+GqQ209L
pMEK
pERK
pp90RSK
Myc tag
EE tag
ERK2
B
AEB
GFP
Gq209L
GRP3wt
GRP3T133A
GqQ209L+GRP3wt
GqQ209L+GRP3T133A
pMEK
MEK
pERK(light)
pERK(dark)
ERK2
pp90RSK
pRasgrp3
pAKT
EE tag
Myc tag
C
GqQ209L+GRP3T133A
GqQ209L+GRP3WT
AEB
AHT
PD
AEB+PD
Aht+PD
pERK
ERK2
pRasGRP3
Myc tag
EE tag
D
T133
REM
CDC25
EF
RasGRP3-ΔC1
T133A
RasGRP3-T133A-ΔC1
GFP
GqQ209L
RasGRP3wt
RasGRP3T133A
RasGRP3wt-ΔC1
RasGRP3T133A-ΔC1
RasGRP3wt+GqQ209L
RasGRP3T133A+GqQ209L
RasGRP3wt-ΔC1+GqQ209L
RasGRP3T133A-ΔC1+GqQ209L
pMEK
MEK
pERK (light)
pERK(dark)
ERK2
pp90RSK
pAKT
AKT
pRasGRP3
Myc tag
EE tag
E
T133
Gly
Cys
myr
pal
REM
CDC25
EF
Fyn-RasGRP3-ΔC1
T133A
Fyn-RasGRP3-T133A-ΔC1
+++
Src-RasGRP3-ΔC1
Src-RasGRP3-T133A-ΔC1
GFP
RasGRP3
RasGRP3-ΔC1
RasGRP3T133A-ΔC1
Fyn-RasGRP3-ΔC1
Fyn-RasGRP3T133A-ΔC1
Src-RasGRP3-ΔC1
Src-RasGRP3T133A-ΔC1
pMEK
MEK
pERK (light)
pERK (dark)
ERK2
pp90RSK
pRasGRP3
Myc tag

Fig.7


Oncogenic GNAQ
PLC β
DAG
PKCi
PKC δ/ε
upregulation
C1
RasGRP3
REM
p
T133
Ras
Raf
MEK
MEKi
ERK

Extended Experimental Procedures

Western blotting antibodies

PKC isoform antibodies used as follows: PKC α(#2056), PKC δ(#2058), PKC ζ(#9368) and PKC ι (#2998) from Cell Signaling Technology; PKC θ (#610090) and PKC ε (#610085) from BD Biosciences; PKC γ (#133800) from Invitrogen; PKC η (#H00005583-B02) from Abnova; PKC βI(sc-209), PKC βII(sc-210), and PKC δ (sc-937) from Santa Cruz; PKC βII(#1514-1) was from Epitomics.

pMEK(#9121), pERK(#4370), SOS1 (#12409), PARP (9542), cleaved PARP(9541), YAP1 (14074), MET(3127), p-MET(3077), Myc tag(2276S) and RasGRP3(#3334) were from Cell signaling technology; GNAQ(E-17), ERK2(c-14), Nras(F155), Kras(sc-30), Hras(sc-520) and Braf(C-19) were from Santa Cruz; β-actin (#A1978) was from Sigma; Glu-Glu(MMS-115P) and HA(MMS-101P) from Covance Inc. (Princeton, NJ, USA); P-p90RSK (#2006-1), RasGRP2 (#S2451) from Epitomics(Burlingame, CA); Human RasGRP1 (JR-E160) was generated by the Roose lab together with Epitomics; RasGRP4 (ab96293) and p-RasGRP3(T133)(ab124823) from Abcam. P-RasGRP1(T184) was generated by the Roose lab through immunization with the peptide SRKL-pT-QRIKSNTC together with Eurogentech/AnaSpec (Fremont, CA).

Quantitative RT-PCR

Total RNA was extracted using PureLink RNA Mini kit (Thermo Fisher Scientific, Waltham, MA). For quantitative RT-PCR, mRNA was retrotranscribed using SuperScript III First-Strand Synthesis Super Mix (Thermo Fisher Scientific). Real-time PCR was performed using standards and Power SYBR Green PCR Master Mix (Thermo Fisher Scientic) with Bio-rad C1000 Touch Thermal Cycler Samples were normalized to a ribosomal protein SB34 with standard comparative CT methods. Primer sequences are shown below: RasGRP3 forward-CTCTGCATGTATCGAAATGCCA; RasGRP3 reverse-

CTACTTCCCGAAATTCCTCAGTC; SOS1 forward-GTAGGATGAACTTGCCCCTG; SOS1 reverse-GCTGCCCTACGAGTTTTTCA.

Genome-wide SNP Genotyping

UM cell line DNAs were genotyped with the illumina Human Omni1-Quad V1.0 beadchip (Illumina, San Diego) according to the manufacture's instructions. The beadchips were processed and imaged on an illumine Bead Array Reader. Bead intensity data obtained for each sample were loaded into Nexus Copy Number software from BioDiscovery (El Segundo, CA) for gene copy number analysis.

Cell fractionation

Subcellular fractionation was performed using the Subcellular Protein Fractionation Kit of Thermo Scientific (Waltham, MA, USA) following the manufacture's instructions. Fractions from cytosolic, membrane and nuclear parts were subjected to western blotting with the indicated antibodies.

Statistical Analysis

Statistical significance was assessed using a standard 2-tailed *t* test using prism 6.0 software (GraphPad software). $P<0.05$ was considered statistically significant.

siRNAs

All siRNAs below were purchased from Dharmacon:

GENE	siRNA description	Catalog number
GNAQ	On-TARGETplus human SMARTpool siRNAs	L-008562-00-0005
PKC δ	On-TARGETplus human SMARTpool siRNAs	L-003524-00-0005
PKC ε	On-TARGETplus human SMARTpool siRNAs	L-004653-00-0005
PKC ζ	On-TARGETplus human SMARTpool siRNAs	L-003526-00-0005
RasGRP1	On-TARGETplus human SMARTpool siRNAs	L-008928-00-0005
RasGRP2	On-TARGETplus human SMARTpool siRNAs	L-009365-00-0005
RasGRP3	On-TARGETplus human SMARTpool siRNAs	L-008517-00-0005
RasGRP3	On-TARGETplus human RasGRP3 individual siRNA-1	J-008517-07-0002
RasGRP3	On-TARGETplus human RasGRP3 individual siRNA-2	J-008517-08-0002
SOS1	On-TARGETplus human SMARTpool siRNAs	L-005194-00-0005
HRAS	On-TARGETplus human SMARTpool siRNAs	L-004142-00-0005
KRAS	On-TARGETplus human SMARTpool siRNAs	L-005069-00-0005
NRAS	On-TARGETplus human SMARTpool siRNAs	L-003919-00-0005
Non targeting control	On-TARGETplus non-targeting siRNA pool	D-001810-10-05
MET	On-TARGETplus human SMARTpool siRNAs	L-003156-00-0005
YAP1	On-TARGETplus non-targeting siRNA pool	L-012200-00-0005
PKC α	On-TARGETplus human SMARTpool siRNAs	L-003523-00-0005

Supplemental Figure Legends

Figure S1. PKC isoforms expression in Uveal melanoma.

A) Validation of specific classical and novel PKC isoform antibodies. 293FT cells were transfected with different HA-tagged PKC wt or constitutively active PKC isoforms cDNAs. After 24h, cell lysates were subjected to Western blotting probed with different PKC isoform antibodies (top panel) and HA antibody (lower panel).

B) Validation of specific atypical PKC isoform antibodies. 293FT cells were transfected with different HA-tagged PKC isoforms full-length cDNAs. After 24h, cell lysates were subjected to Western blotting probed with indicated antibodies.

C and D) The effect of knockdown of GNAQ and PKC isoforms on MAPK signaling of levels of pMEK and pERK in two uveal melanoma cell lines with GNAQ mutations (OMM1.3 and mel202), one UM cell line with GNA11 mutation (UPMD-2) and two GNAQ/11 wild type cell lines (mum2c and mel285 from uveal origin)(D). Cells were transfected with respective siRNAs either singly or in combination and then lysed.

E)The effect of siRNA mediated knockdown of PKC βII on pERK levels in two UM cell lines with GNA11 mutation. As noted, PKC βII is knocked down well in OMM-GN11 cells but undetectable in UPMD-2 cells. -: no siRNA. NT: non-targeting siRNA.

Figure S2 PKC δ and PKC ε are important downstream effectors of oncogenic GNAQ in mediating MAPK signaling.

A) kinase dead mutants of PKC δ and ε significantly impaired to activate MAPK with oncogenic GNAQ. 293FT cells were transfected with indicated plasmids for 24h and analyzed for indicated protein expression with immunoblot. pMEK, pERK and p-p90RSK as MAPK signaling readout. PKC isoforms expression was detected with HA tag. $GNAQ^{Q209L}$ was detected with Glu-Glu tag. δ^{kd} and ε^{kd}

mean kinase dead mutant of PKC δ and PKC ε, respectively. A representative western blot was shown in left panel. Quantification of three independent western blots for pMEK, pERK and pp90RSK were shown in right panel. Data normalized to cells expressing GFP. Value represents mean± s.e.m of the three independent experiments.

B)Oncogenic GNAQ not wild type GNAQ synergistically activate MAPK with wild type PKC δ and PKC ε. 293FT cells were transfected with indicated plasmids for 24h and analyzed for indicated protein expression with immunoblot.

C) knock down of pkc isoform α after 7 day siRNA transfection. UM Cells with GNAQ or GNA11 mutation were transfected with indicated siRNAs as indicated. After 7 days, cell were lysed and subjected to western blotting.

D) knock down of pkc isoforms after 7 day siRNA transfection**.** Melanoma Cell lines with GNAQ wt were transfected with indicated siRNAs as indicated. After 7 days, cell were lysed and subjected to western blotting.

E) Western blots of subcellular fractions of two different normal human melanocytes (NHM-1 and NHM-2) probed with indicated antibodies. Notes: PKC α and PKC δ are localized in cytoplasmic fractions. C: Cytoplasmic; M: Membrane; N:Nucleus.

F) Western blots of subcellular fractions of 5 UM cell lines with GNAQ/11 mutations probed with indicated antibodies. As noted, PKC α is localized in cytoplasmic fractions, while PKC δ is localized in both cytoplasmic and membrane fractions.

G) Western blots of subcellular fractions of TPA starved mouse melanocytes (Melan-a) treated with or without 200 nM TPA for indicated times. As noted, TPA induced the membrane translocation for PKC α, δ, ε but not ζ.

H) $GNAQ^{Q209L}$ combined with PKC δ increased more Ras-GTP levels than with PKC α and PKC z. in 293FT cells. 293FT cells were transfected with indicated cDNA plasmids for 24h. Ras-GTP pull-down was performed and detected by Western blot with a pan-Ras antibody. Expression levels of $GNAQ^{Q209L}$ were

monitored with Glu-Glu (EE) tag. PKC δ ,PKC α and PKC ζ were detected via HA tags.

I) Knock down of Ras inhibited the proliferation of UM cell line Omm1.3 . Omm1.3 cells were transfected with non-targeting (NT) or all three Ras isoform siRNAs. 48h after transfection, cell were counted and plated at equal density. Cells were counted at day 1, 3, 5 after plating.

Figure S3 RasGRP3 is markedly upregulated in both GNAQ and GNA11 mutant melanoma cells.

A) Quantitative RT-PCR shows no expression difference in RasGEFs (RasGRP1, RasGRP2, RasGRP4 and SOS1) between human GNAQ/11 mutant UM cell lines and GNAQ/11 wt cell lines.

B) **Validation of specific RasGRP isoform antibodies.** 293FT cells were transfected with GFP, $GNAQ^{Q209L}$ and myc tagged RasGRP isoforms cDNAs . 24h later, cells were lysed and subjected to western blotting probed with different RasGRP isoform antibodies.

C) western blotting of GNAQ and GNA11 mutant melanoma cell lysates and human melanocyte lysates . IHM: immortalized human melanocytes. NHM-1 and NHM-2 : normalized human melanocytes

D) Both RasGRP3 protein and phosphorylation levels are much higher in GNAQ mutant melanoma cells (92-1, omm1.3, mel270 and mel202) compared to Braf mutant melanoma cells (A375, SK-MEL-5, UACC257 and MALME-3M all from cutaneous melanoma) and Ramos cells.

E) No detectable expression of RasGRP3 in CRMM1 and CRMM2 conjunctival melanoma cell lines without GNAQ/11 mutations compared to GNAQ/11 mutant UM cell lines (OMM1, 92-1, mel270, mm2.5 and omm2.3) with western blotting.

F) Positive RasGRP3 IHC staining in OMM1 UM cell lines with GNA11 mutation. Images were taken at magnifications 20X.

Figure S4 PKC signaling regulates RasGRP3 expression and phosphorylation in uveal melanoma cells.

A) PKC inhibitor AEB071 decreased RasGRP3 expression and phosphorylation in 92-1 cells in a time dependent manner. Omm1.3 cells were incubated with DMSO or 500nM AEB071 for the times indicated.

B) Both PKC inhibitor AEB071 (top panel) and AHT956(bottom panel) decreased RasGRP3 expression and phosphorylation in 92-1 and mel202 cells in a dose dependent manner. 92-1 and mel202 cells were incubated with DMSO or AEB071 or AHT956 at indicated doses for 24h.

C**)** PKC inhibitor AEB071 has no effect on RasGRP3 expression in SK-MEL-5 with Braf mutation and MM485 with Nras mutation melanoma cells. SK-MEL-5 and MM485 were incubated with AEB071 at different dosage for 24 h.

D) TPA increased RasGRP3 expression and phosphorylation in human melanoma cells with Braf mutation. SK-MEL-5 and UACC257 cells were incubated with or without 200nM TPA for 72h.

E) TPA increased RasGRP3 expression in human melanocytes. Immortalized human melanocytes were incubated with or without 200nM TPA for the indicated times. Quantification of three independent western blots for RasGRP3 and SOS1 were shown. Data are normalized to cells without TPA treatment. Value represents mean± sem of the three independent experiments. As noted, 2h and 6h TPA treatment increased SOS1 levels but not statistically significant.

F) PKC δ and PKC ε increase T133 phosphorylation of RasGRP3 in 293FT cells. 293FT cells were transfected with indicated cDNAs for 24h. Quantification of two independent western blots for p-RasGRP3 was shown. The p-RasGRP3 level in RasGRP3 cDNA transfection alone is normalized to total RasGRP3 level and regarded as 1.

G) The MEK inhibitor Trametinib has no effect on RasGRP3 expression and phosphorylation in GNAQ mutant cells. 92-1 and Omm1.3 cells were incubated with Trametinib at different doses for 24h.

Figure S5 both YAP and MET pathways do not contribute to RasGRP3 upregulation and MPAK signaling in UM cells with GNAQ mutations.

A)siRNA mediated knockdown of Yap1 has no effect on RasGRP3 expression and pERK levels in both 92-1 and Omm1.3 cells. Cells were transfected with no siRNAs(-), non-targeting siRNAs (NT) and YAP1 siRNAs for 72 hrs and lysed for western blotting.

B) YAP1 inhibitor Verteporfin did not inhibit MAPK signaling in 92-1 UM cells. 92-1 cells were treated with DMSO, AEB071(250nM), Verteporfin (0.1ug/ml), Verteporfin (0.5ug/ml), AEB071(250nM)+Verteporfin(0.1ug/ml) and AEB071 (250nM)+Verteporfin(0.5ug/ml) for 24h and lysed, probed with indicated antibodies. AEB: AEB071. Vert: Verteporfin. CI-PARP: cleaved PARP.

C) Combined inhibition of YAP1 and PKC/MAPK pathway has no synergy to inhibit MAPK pathway and induce apotosis in 92-1 cells. 92-1 cells transfected with non targeting siRNA (NT) or YAP1 siRNA were treated with DMSO, AEB071 (250nM), MEK162 (50nM) or AEB071+MEK162 for 24h.

D) MET knockdown did not affect both RasGRP3 and p-RasGRP3 levels in two UM cells with GNAQ mutations. 92-1 and Mel270 cells were transfected with no siRNA(-), non-targeting siRNAs (NT) or MET siRNAs for 72 hrs and subjected to western blotting.

E) MET inhibitor has no effect on both RasGRP3 and p-RasGRP3 levels on UM cells with GNAQ mutations. 92-1 and Omm1.3 cells were treated with DMSO or 250 nM INC280 in the presence or absence of 10ng/ml HGF for 24h. As noted, INC280 inhibited pMET in the presence or absence of the MET ligand HGF.

F) Combined inhibition of PKC/MAPK and Yap pathway or PKC/MAPK and MET pathway has no synergy in inhibiting proliferation of UM cell lines. Omm1.3 and 92-1 cells were treated with DMSO, AEB071 (250 nM), INC280(250nM) and MEK162 (50nM) singly or in combination for 14 days. Cells were stained with Crystal Violet.

Figure.S6 RasGRP3 is regulated transcriptionally in GNAQ mutant UM cells.

A and B) No gene amplification for RasGRP3, PKC ε and PKC δ in 92-1 (A) and mel202 (B) UM cells. Extracted cell DNAs are subjected to SNP genotyping. The gene copy number was analyzed with nexus copy number software. The copy number change and Loss of heterozygosity for the whole genome (left panel) or indicated genes (right panel) are shown.

C) No RasGRP3, PKC δ and PKC ε amplification in human uveal melanoma tissues. The data are derived from uveal melanoma TCGA project and www.cBioportal.org.

D) RasGRP3 gene alteration across cancers. The data are from TCGA projects and the plot is from cBioportal for Cancer Genomics (http://www.cbioportal.org/index.do)

E) Knock down of GNAQ decreased RasGRP3 mRNA but not SOS1 in both omm1.3 and 92-1 cells. Cells were transfected with non-targeting siRNAs (NT) or GNAQ siRNA for 72 h and lysed for RNA extraction and quantitative RT-PCR. Data are mean ± sem of two to three independent experiments.

F) Overexpression of $GNAQ^{Q209L}$ and $GNA11^{Q209L}$ in human melanocytes increased RasGRP3 mRNA levels. Human melanocytes were infected with lentivirus expressing GFP, $GNAQ^{Q209L}$ or $GNA11^{Q209L}$. After selection with blasticidin, RNAs were extracted for RasGRP3 quantitative RT-PCR. Data are mean ± sem of two independent experiments.

G) PKC inhibitor decreased RasGRP3 mRNA significantly in both 92-1 and Omm1.3 cells. Cells were treated with 1uM AEB071 or 1uM AHT956 for 24h. RNAs were extracted for quantitative RT-PCR. Data are mean ± sem of two independent experiments.

H)TPA increased RasGRP3 mRNA in both human melanocytes and UACC257 melanoma cell lines with BRAF mutation. Cells were treated with 200nM TPA for 48h. Value represent mean ± sem of two independent experiments.

Figure S7 RasGRP3 is functionally important for uveal melanoma cells harboring GNAQ mutations

A) Quantification of three independent western blot experiments as shown in Fig.5A. pMEK, pERK and pAKT levels were normalized to total MEK, ERK2 and AKT respectively.

B) RasGRP3 knock down significantly diminished MAPK signaling in UM cells with GNAQ mutation. 92-1 and omm1.3 cells were transfected with two different individual RasGRP3 siRNAs as well as no siRNA(-) and non-target siRNA (NT) controls for 72h.

C) Only RasGRP3 knock down but not other RasGRP isoform knock down diminishes MAPK signaling in omm1.3 cells. Omm1.3 cells were transfected with indicated siRNAs for 72h and lysed for western blotting.

D) Only RasGRP3 but not SOS1 knock down significantly decreased MAPK signaling in 92-1 cells. 92-1 cells were transfected with indicated siRNAs for 72h and lysed for western blotting.

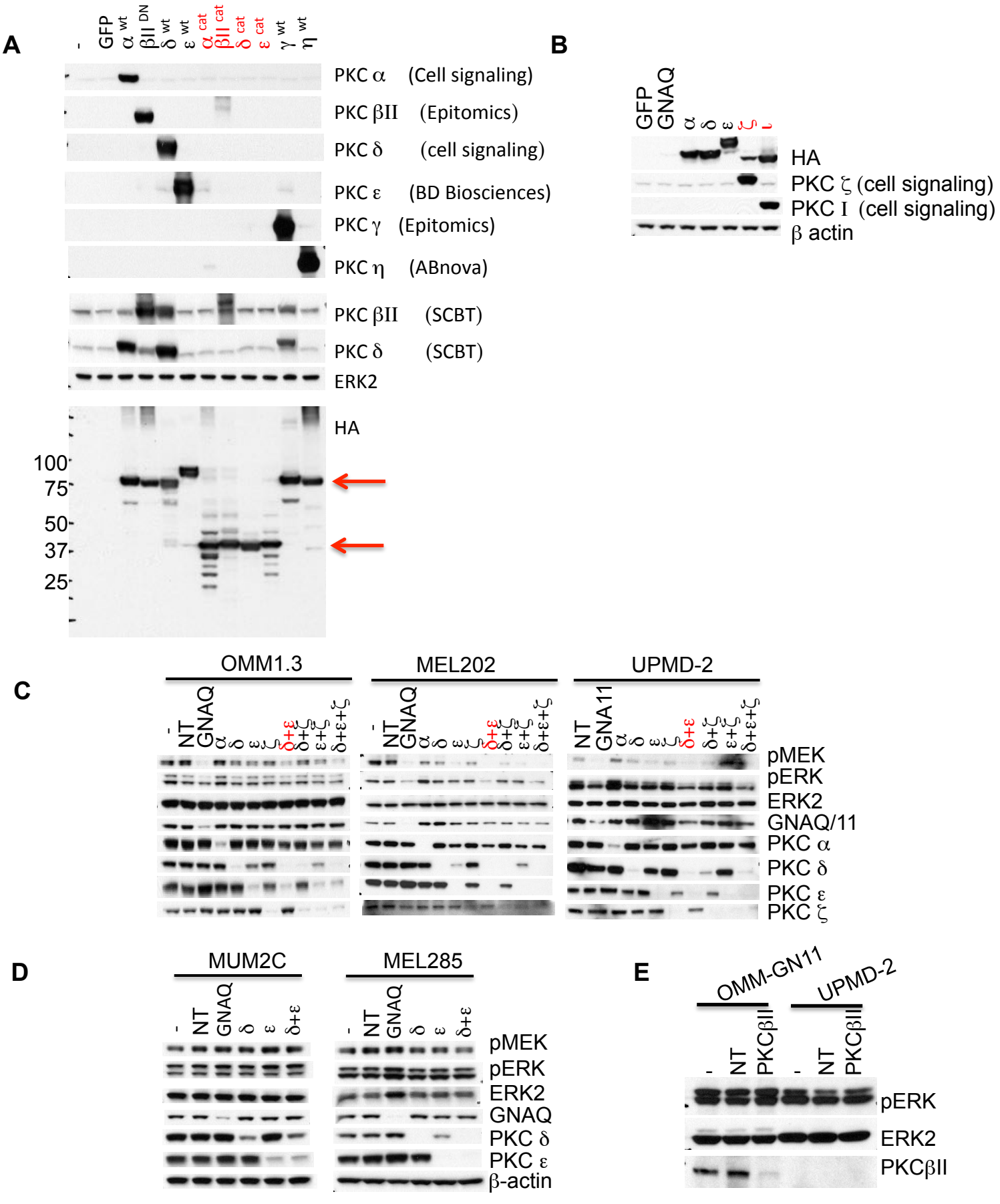
E) Knock down of GNAQ and RasGRP3 decreased Ras-GTP levels in omm1.3 cells. Omm1.3 cells were transfected with non-targeting siRNAs(NT) , GNAQ and RasGRP3 siRNAs for 72h and lysed for western blot.

F) knock down of pkc isoforms after 7 day siRNA transfection. SK-MEL-5 and UACC257 melanoma cells were transfected with non target (NT) siRNAs and RasGRP3 siRNAs. After 7 days, cell were lysed and subjected to western blotting.

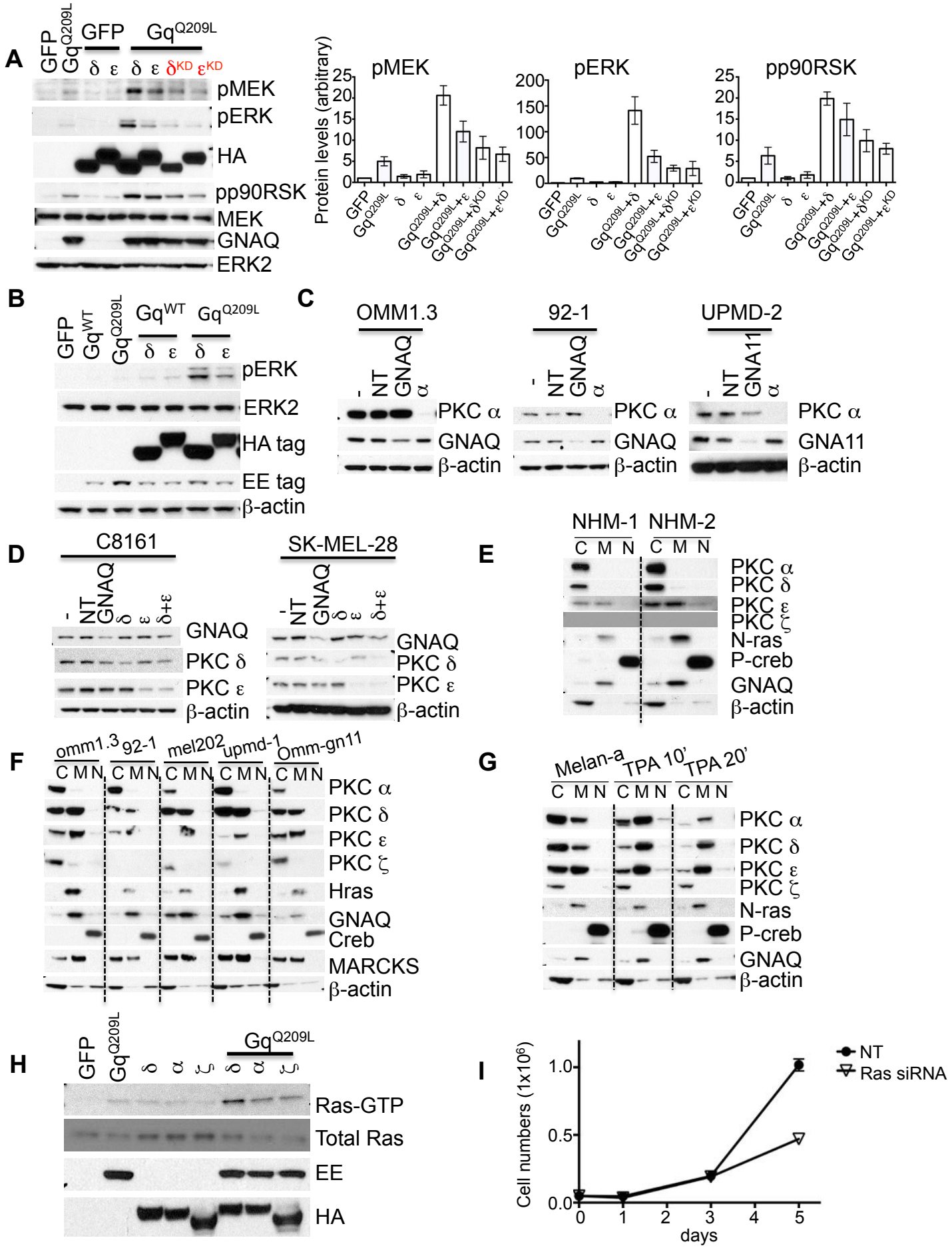
G) RasGRP3 synergizes with $GNAQ^{Q209L}$ in activating Ras in 293FT cells. 293FT cells were transfected with indicated plasmids for 24h. Activated Ras was detected. $GNAQ^{Q209L}$ was probed with EE-tag. Myc tag was used to detect RasGRP3 or $RasGRP3^{T133A}$ expression.

H) PKC isoforms expression in 293FT cells compared to UM cell line UPMD-1.

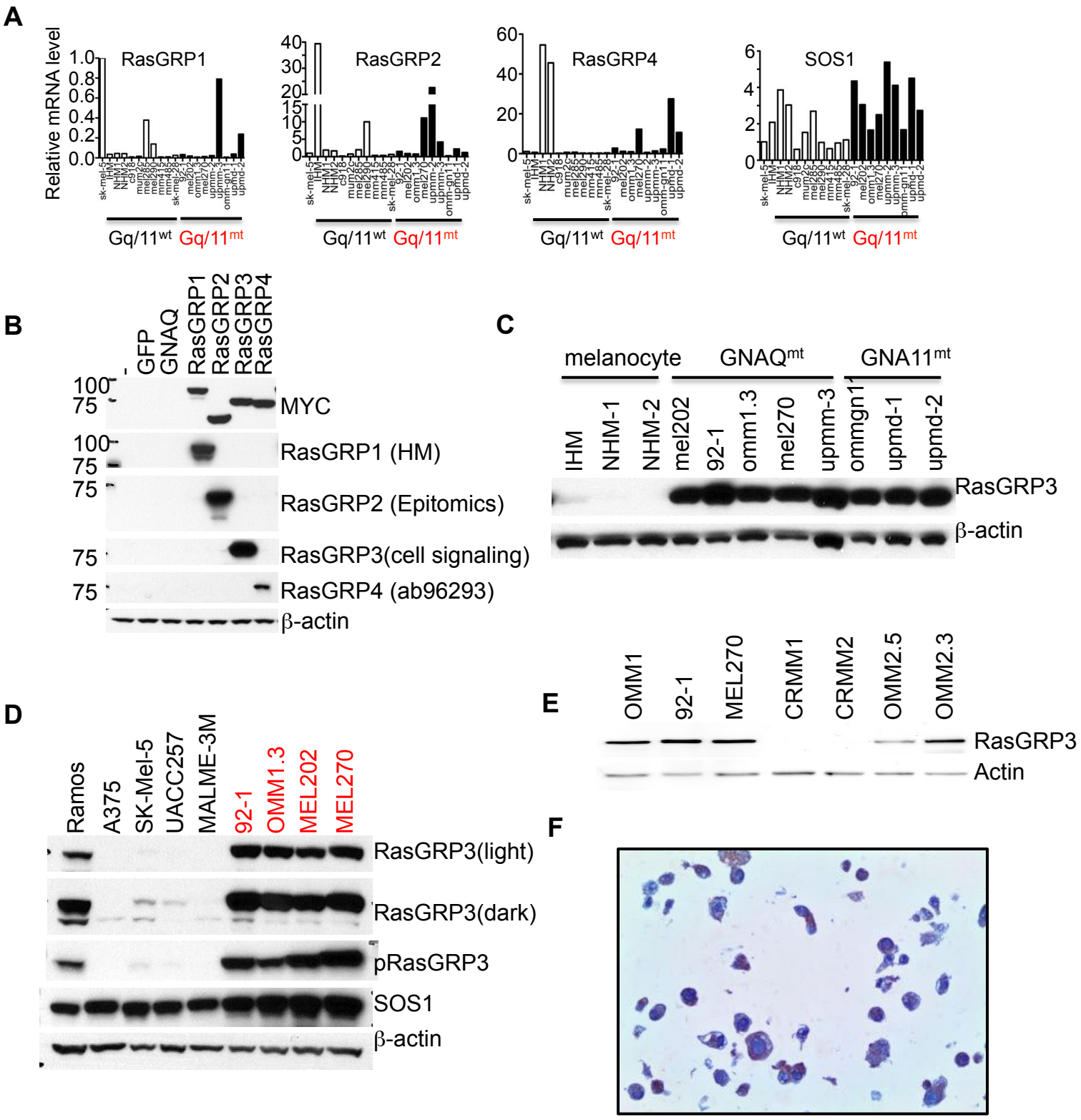
Supplemental Fig.1



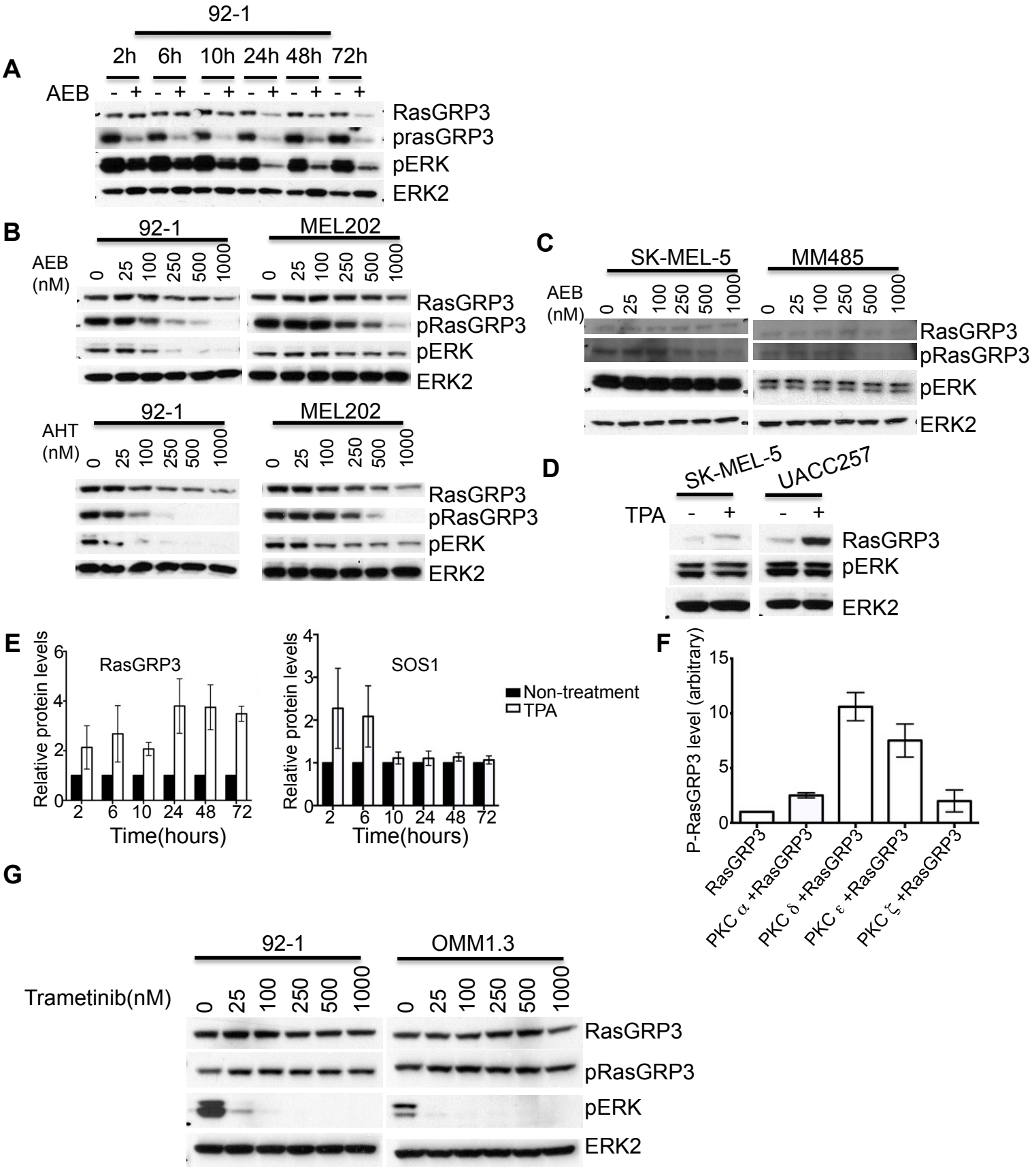
Supplemental Fig.2



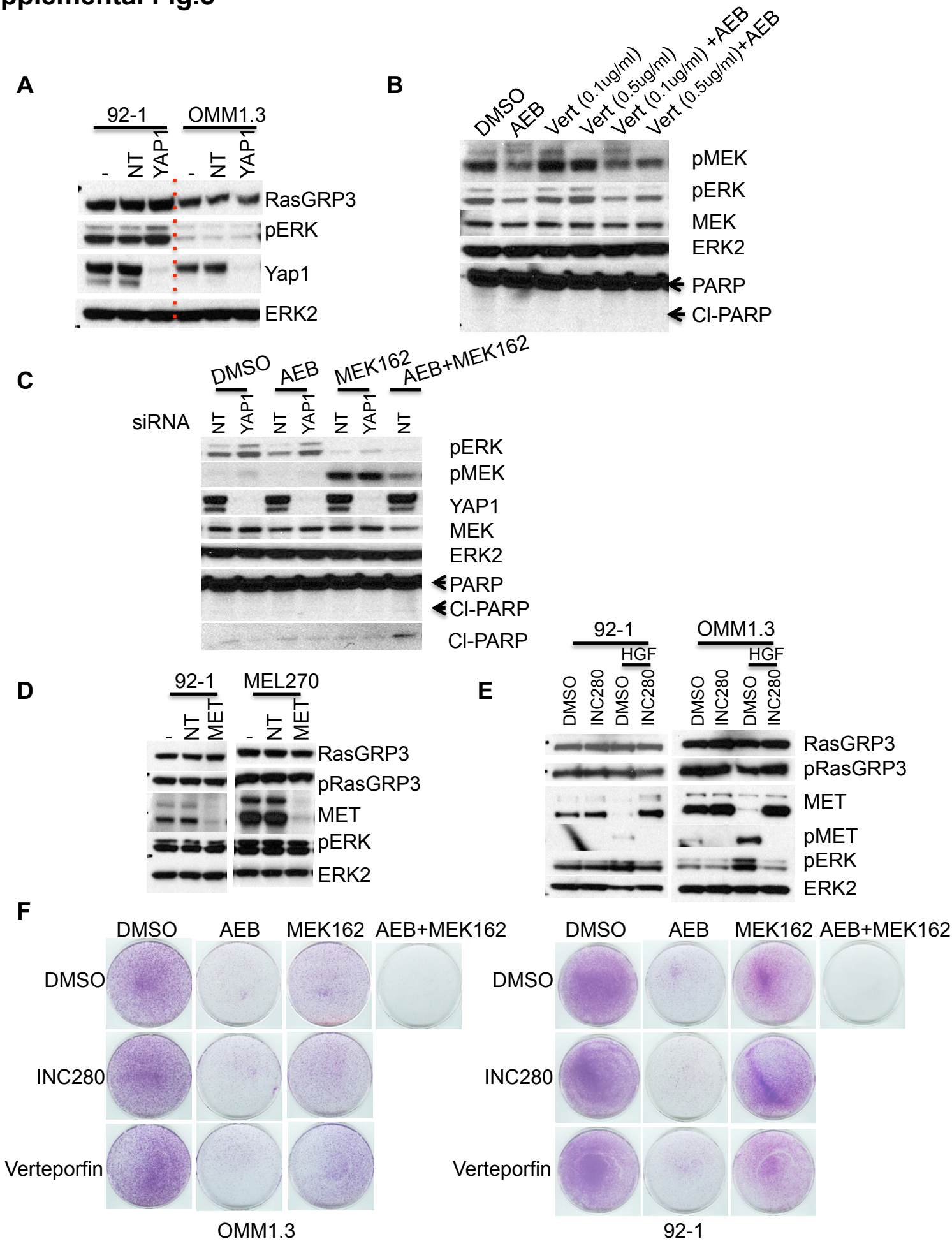
Supplemental Fig.3



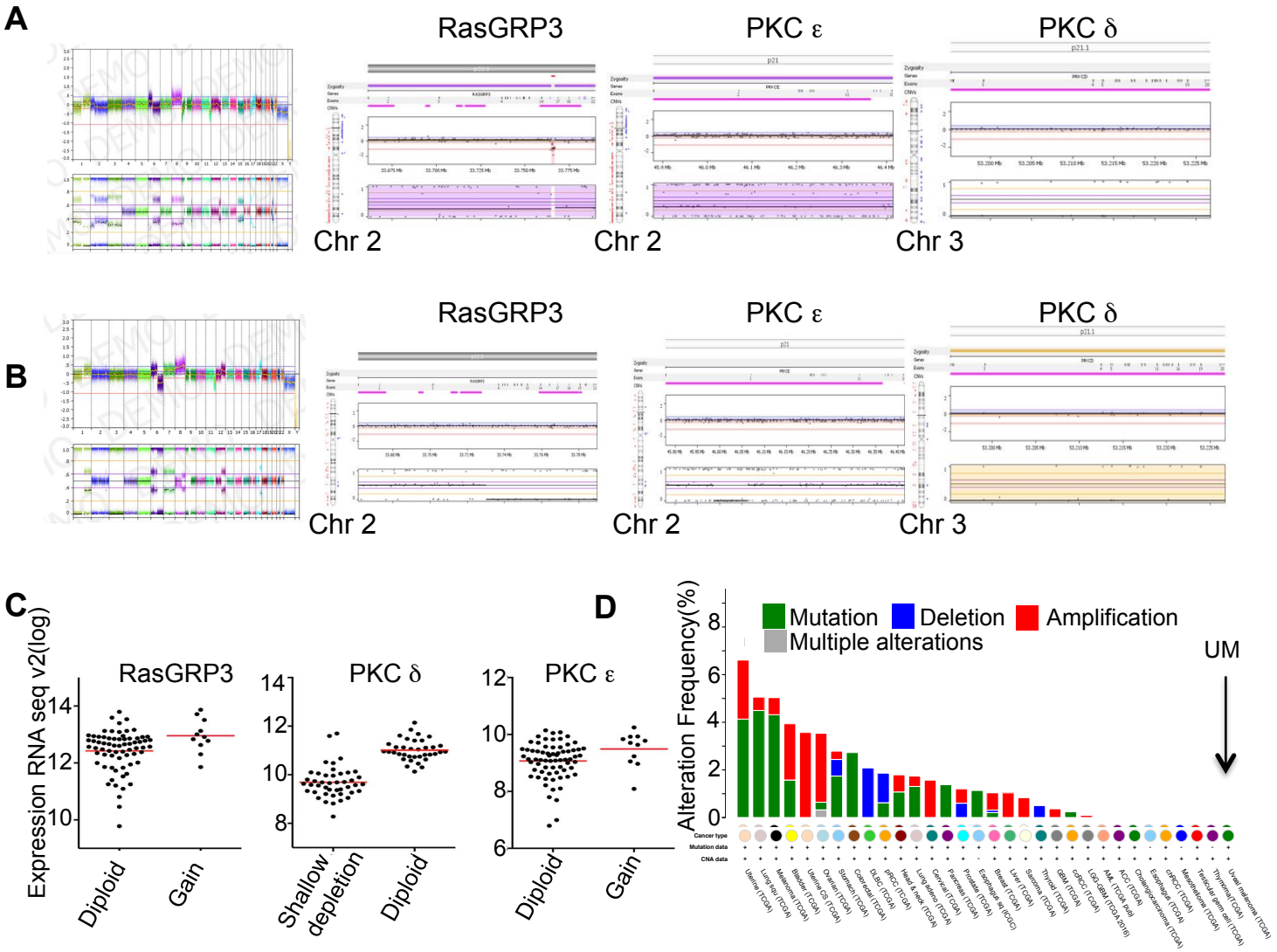
Supplemental Fig.4



Supplemental Fig.5



Supplemental Fig.6



Supplemental Figure 7